Factors influencing follicular development in mammalian ovaries.

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Abstract

The studies described in this thesis have been concerned with several aspects of follicular development in the mammalian ovary. Chapters 2, 3 & 4 deal with mathematical modelling of ovarian follicle dynamics in normal animals and comparisons with experimentally manipulated animals. Chapter 5 describes a novel method for estimating the clonal origin of the mouse ovarian follicle. In the final two chapters (6 & 7) the comparative physiology and anatomy of follicular numbers and sizes and the incidence of polyovular follicles are described for a number of species. The unifying theme of these studies is that they reveal patterns existing in follicular development and utilisation by detailed examination of one species, (CBA/Ca mouse), and broadly by interspecific comparisons (with relation to scaling).

A detailed mathematical description of the follicular dynamics of virgin CBA/Ca mice up to 98 days of age has been obtained by the application of compartmental modelling to differential follicle counts. The rates of follicle growth (migration) and death have been estimated for five stages of development (primordial to Graafian). The model predicts age changes in follicle growth and death rate, there being transitions in the parameters at 20 days second at 60 days. The parameters for normal animals have been compared with those of animals under two experimental conditions: 1) by unilateral ovariectomy at 42 days of age, which abruptly halves the numbers of ovarian follicles and alters the ratio of large : small follicles. 2) by blocking ovulation using progesterone implants. The dynamics of follicle growth were altered by both treatments in comparison with the controls. Follicles at all stages of development were affected by unilateral ovariectomy and differences

may exist with time. The compensatory response by the remaining ovary was due to a combination of an increased preantral growth rate and a decrease in atresia at antral stages. Earlier stages of follicle development were affected this may have been incidental to the compensatory response. In progesterone treated animals follicles developed through to antral stages when they underwent atresia. The effects of treatment were observed at three levels of development: 1) The initiation of growth from the primordial pool, 2) Growth rate of small follicles and 3) deaths at larger stages of follicular development. Longer term observations indicated that these effects may not be constant.

The modelling studies have looked at numerical changes in the follicle population with time but a greater understanding of the develomental biology of the follicle is required in order to explain the changes in growth and death rates observed. This problem has been tackled initially by studying the clonal origin of the follicular epithelium. The technique used is based on the principle that cells in females are generally mosaic as a result of X-chromosome inactivation the use of X linked cell markers phospho-glycerate kinase-1 (PGK-1). Granulosa cells were found to be polyclonal in origin with the number of progenitor cells numbering 5 on average. Analysis of cumulus and mural granulosa cells showed that substantial cell mixing had occurred and cumulus cells were generally founded by more than one clone.

Finally, comparative studies have been conducted to look at scaling of follicle sizes and numbers and of polyovular follicles. Ovarian follicle and oocyte sizes were scaled according to body weight (ranging from .005-500Kg) using data from 22 species. Primordial and Graafian follicle

sizes varied with body weight but closer correlations for the latter were obtained when the sum of the surface areas or volumes for a preovulatory set were considered as opposed to the values for individual follicles. The numbers of nongrowing follicles in reserve at young adult ages were correlated with maximum longevity of the species and related to body weight. The frequency of polyovular follicles varied in species studied and were most abundant in the domestic bitch. The overall incidence of polyovular follicles in young bitches was 14 %, being reduced to 5% in bitches at 7-11 years. The frequency of the various types of polyovular preantral follicle varied inversely with the numbers of oocytes per follicle.

Declaration:

I declare that this thesis is my own composition and that the work described in it was carried out by me except where acknowledgement is made to the contribution of others (see below).

Computation of the mathematical model (chapter 2, 3 & 4) was carried out by a statistician Dr Malcolm Faddy whilst at the University of Birmingham. Electrophoresis for detection of PGK (chapter 5) was carried out by Dr J.D. Ansell of the Department of Zoology University of Edinburgh. The Progesterone assays (chapter 4) were carried out by Dr Hamish Fraser of the MRC Reproductive Biology unit. Dr R.G. Gosden of the Department of Physiology University of Edinburgh assisted with counts of primordial follicles (chapters 2, 3 & 4) and with follicle measurements (chapter 6).

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Chapter One:
General Introduction.

General Introduction:

This thesis is concerned with identifying patterns in follicular development and examining some of the factors influencing them. The follicular dynamics of the mouse are examined in detail but other aspects of follicular development have been studied in several species and a cross species comparison obtained. This introduction will look at some aspects of the ovarian system and highlight some of the existing gaps in our knowledge with respect to this system and some of the questions that have been tackled in this thesis.

The mammalian ovary:

The mammalian ovary has two major functions: the production of gametes and the synthesis of hormones. The basic developmental unit in the ovary is the follicle. Follicles begin their developmental history at primordial stages in which a small oocyte is enclosed by a single layer of squamous cells. When mature they comprise of a large oocyte, enclosed by a granulosa (epithelial) cell membrane and an envelope of theca (stroma) cells in intimate association. Since follicle development is the theme of the studies to be described it is appropriate to describe in outline the process of oogenesis and follicle formation.

Origin of germ cells:

The origin of mammalian germ cells was the subject of scientific debate for many decades based on two main viewpoints namely that of Waldeyer (1870), who proposed that germ cells were formed during embryonic development through proliferation of the so called germinal epithelium. The main exponents of the second viewpoint were Goette, (1875); Balfour, (1878) and Nussbaum, (1880), with this being that germ cells

became segregated from somatic cells before the formation of the organ systems of the embryo and that a continuous germ cell lineage exists through successive generations. The debate continued into the middle of the 20th century with opinion ranging from the denial of a germinal nature of the so-called primordial cells (Simkins, 1923) to acceptance of the germ line hypothesis (Okkelberg, 1921; Nieuwkoop, 1949). It is now agreed that primordial germ cells have an extra-gonadal origin and that they migrate from the wall of the yolk sac to the germinal ridge (Witschi, 1948) where they undergo rapid multiplication by mitosis. At this latter stage the germ cells are described as oogonia.

Whether or not the numbers of germ cells were fixed early in life or capable of continuing formation in adult life was not settled until the 1950's. In the 1920's the general view was that the oocyte supply was fixed (Pearl & Schoppe, 1921) but this was challenged by Allen, (1923) who believed he had evidence to support the theory that the formation of oocytes (oogenesis) continued throughout reproductive life. It was proposed that cyclical proliferation of the germinal epithelium gave rise to oocytes at each oestrous/menstrual cycle (Allen, 1923; Evans & Swezy, 1931; Allen & Creadick, 1937). This was a widely held view until the 1950's when Zuckerman (1951) showed by extensive studies based on differential counting of follicles that oocytes were not produced throughout lifespan in most mammals. Further evidence using tritiated thymidine labelling of oocyte nuclei have confirmed that oocytes in juvenile and adult ovaries are direct descendants of the fetal germ cells (oogonia) and that the germ cells do not increase in number throughout life (Rudkin & Griech, 1962; Borum, 1966; Peters & Crone, 1967). There is evidence that in some species of the family Lemuroida

that oogenesis continues in adult life (Anand Kumar, 1968; David *et al.*, 1974), but this is exceptional.

Oogenesis:

At some time during fetal development (or in some species shortly after birth) oogonial mitoses ceases and germ cells enter meiosis, the cell products are termed oocytes. Once meiosis has been initiated it progresses to prophase of the first meiotic division and then germ cells enter an arrested phase of development and no further nuclear maturity occurs until preovulatory or atretic changes occur in the large follicle (Baker, 1963). This arrested phase could last several years or decades depending on the species and has often been referred to as a resting phase (dictyate) however, it is known that at this time the oocyte is actively synthesising ribonucleoprotein (Baker & Franchi, 1967 a,b,c.; Baker *et al.*, 1969; Baker, 1971; 1972). Thus, although meiosis is arrested in the primordial oocyte it should not be considered a 'resting stage'. Many oocytes degenerate during meiosis and it is thought that this is most marked during zygotene or pachytene (Beaumont & Mandl, 1962); it has been estimated that as little as 5% of the maximum numbers of germ cells in the fetal human ovary reach the diplotene stage (Baker, 1963).

The long dictyate period has been suspected to be the cause of various forms of chromosomal imbalance (Edwards, 1980). To explain the high incidence of abnormalities with ageing it was postulated that an increased frequency of chiasmata occurred with aging indicating that the first formed oocytes were the first used. This led to the first in first out hypothesis or 'production line hypothesis', (Henderson &

Edwards, 1968; Edwards, 1970). This theory is based purely on genetic evidence (Luthardt *et al.*, 1973; Polani & Jagiello, 1976; Speed, 1977).

The experiments of Byskov and her collaborators on inductive tissue interactions within the developing gonad have provided insight into the mechanisms that may trigger the initiation and arrest of meiosis, (Byskov, 1975; 1978a,b). They suggested that the signal for the initiation of meiosis originated from the mesonephric connections to the gonad, i.e. the rete cords, and that this system secretes a diffusible substance, meiosis inducing substance, and that a second factor probably produced by the granulosa cells counters the action of the first substance by inhibiting meiosis. In transplantation experiments it was shown that in the absence of rete, meiosis was not induced (Byskov, 1975; O & Baker, 1976). Thus, it was suggested that the interaction between the two substances regulated meiosis of the developing gonad.

The exact mechanism by which the initiation of meiosis is triggered is still not known and it is probably a combination of cellular contact and humoral substances (see Byskov, 1986). Whatever the mechanisms they result in the postnatal ovary containing a large pool of non-proliferating oocytes (Richards, 1980).

Formation of follicles:

Immediately after the first oocytes reach the diplotene stage follicle development begins and this always starts in the inner part of the ovary (Mossman & Duke, 1973). The process by which the oocyte is enclosed by layers of somatic cells is known as folliculogenesis. A

follicle is formed when the presumptive granulosa cells surround the oocyte and an intact basal lamina encloses the unit (Peters, 1978). What causes the oocyte population to form follicles is not known .

The presence of an oocyte is required for the formation of follicles. Follicles fail to form in sterile ovaries e.g. Turner syndrome (Weiss, 1971; Peters 1978) and in cases of experimental destruction of oocytes no follicles form (Vanhems & Bousquet, 1973; Merchant-Larios, 1976). The effect of extra and intra ovarian factors on follicle formation is still unknown. Culture of hamster ovaries at the time of follicle formation with no additional gonadotrophins revealed retarded follicular development and differences in the granulosa cells (Challoner, 1975). Evidence on the role of gonadotrophins and other factors on follicle formation is equivocal and indeed in hypogonadal mice (which are deficient in gonadotrophin releasing hormone (GnRH) with a consequent reduction in pituitary and plasma gonadotrophins) follicles are formed. Follicle formation is also dependent upon the presence of the rete ovarii as implied above (Byskov, 1974a; Byskov *et al.*, 1977).

The origin of the somatic cells of the follicle is not certain, and many tissues in the early ovary have been implicated as progenitors of granulosa cells (for a detailed review of the embryonic development of the ovary see, Byskov, 1986). Embryonic cells of the stroma (review, Mossman & Duke, 1973), the rete ovarii (Byskov & Lintern-Moore, 1973; Byskov & Rasmussen, 1973) and surface epithelium (Merchant-Larios, 1979) have been suggested as originators of granulosa cells. Folliculogenesis always occurs first in the innermost part of the ovarian cortex, in that part of the ovary where the oocytes come into

intimate contact with the rete ovarii. It has been suggested that the rete cells move between the oocytes and become attached to their surface (Byskov, 1975). The functional significance of the close relationship between the rete ovarii and oocytes was shown in the mouse, ferret, cat and mink ovary (Byskov & Lintern-Moore, 1973; Byskov, 1975). A function for the rete ovarii in the origin of follicle cells was ascribed as early as 1898 by Kölliker as he observed that the cells of the follicular envelope were continuous with cord cells. It may be that the origin of the granulosa cells is species dependent. Granulosa cell - rete associations have been observed in many species: cat, mink, ferret, mouse (Byskov, 1975; Byskov & Lintern-Moore, 1973; Byskov & Rasmussen, 1973) and in the rat (Stein & Anderson, 1979). Cells of the surface epithelium probably have a more important role in other species such as the human, (Motta & Makabe, 1982). What is unclear is the number of cells that are necessary to be present before a follicle is formed and whether only one cell divides or several cells are involved in this process.

Polyovular follicles:

Follicles with more than one oocyte enclosed in the follicular envelope have been observed in many species. These structures have been observed in man, monkey, dog, cat, ferret, pig, sheep, goat, rabbit, mouse, rat, guinea pig, prairie dog, bat, armadillo, kangaroo, native cat and opossum (Hartman, 1926; Brambell, 1956) and several other species. The incidence of these structures seems to vary considerably between species although precise quantitative studies have not been conducted. Presumably polyovular follicles are formed during folliculogenesis but it remains unclear why their incidence varies so much between species.

It has also been assumed by a number of authors that polyovular follicles are abnormal structures usually confined to prepubertal animals (Harrison & Weir, 1977).

Follicle growth:

Once folliculogenesis has been completed a pool of small follicles forming a non-renewable source of germ cells within the ovary exists this is often referred to as the primordial pool. The growth of follicles begins with the enlargement of the primary oocyte (still arrested in meiotic prophase) and then successive divisions of the cuboidal follicle cells giving rise to a multilayered lining of granulosa cells and eventually theca cells organise as a layer external to the basement membrane. The oocyte is separated from the granulosa cells by the zona pellucida. Protoplasmic processes from the adjacent granulosa cells traverse the zona pellucida and interdigitate with microvilli arising from the oocyte (see reviews, Baker, 1972; Zamboni, 1974). Follicle cell gap junctional complexes have been found along macrovilli that penetrate the oocyte cytoplasm and tightly fuse the two cells together (Gilula *et al.*, 1978; Dekel *et al.*, 1978; Browne *et al.*, 1979). Presumably a transfer of nutrients to the oocyte occurs by diffusion (Zamboni, 1974) or by pinocytosis of granulosa cell projections (Hope, 1965; Baker, 1972).

Continued proliferation of the granulosa cell layers (membrana granulosa) results in a fluid-filled space, known as the antrum, forming within the follicle. The formation of an antrum occurs in the ovarian follicles of most mammals with an exception being the Tenrec (Nicoll & Racey, 1985). It is not known whether the formation of an antrum is

imposed by some physical limitation of size. Large amounts of follicular fluid can accumulate within the antral cavity and this fluid is presumed to consist of a mixture of transudates of plasma and granulosa cell secretions (Edwards, 1974; McNatty, 1978). As a consequence of the formation of an antrum two types of granulosa cells can be identified; mural granulosa cells, those cells forming the lining of the antral cavity and cumulus cells, those cells surrounding the oocyte. Studies have demonstrated that these two cell populations are functionally distinct, mural granulosa cells have been shown to secrete plasminogen activator and glycosaminoglycans but cumulus cells do not (Gilula *et al.*, 1978; Yanagishita *et al.*, 1979). The differences observed between cumulus and mural granulosa cells raises questions as to their origin. Both cell types are derived from cells of common embryological origin but are there clones of cells that are predetermined to become a particular type? Further knowledge of the developmental biology of ovarian follicles is required in order to tackle these questions.

The number of developmental stages a follicle goes through from primordial to Graafian is unknown but classification systems have been devised to describe the continuum of follicle development in terms of stages normally based on oocyte size and number of granulosa cell layers. Such classification systems have been designed for convenience and the morphological criteria need not have a biological basis. Two widely used classification systems are those of Pedersen & Peters (1968) and Mandl & Zuckerman, 1951. In the studies described in this thesis the system of Mandl & Zuckerman 1951, has been used.

Initiation of follicle growth:

The age at which follicle growth is initiated varies in different species, with it beginning during foetal life in cow, monkey and human and in the perinatal period in mouse, rat, mink, ferret, rabbit and hamster (Lintern-Moore, 1972; Peters, 1978). Follicle growth is initiated continuously irrespective of the animals physiological status (Pedersen, 1970) but the hormonal or other factors regulating these events are unknown. There is some doubt as to whether gonadotrophins are required for initial growth. Evidence exists to suggest that gonadotrophins are required for "priming" in the neonatal ovary before follicle growth can begin (Baker & Scrimgeour, 1980) but, preantral growth occurs in hypophysectomised animals (Faddy *et al.*, 1976) and in those treated with gonadotrophin antisera (Eshkol & Lunenfeld, 1972).

Follicular growth is continuous until death (atresia) or ovulation occurs (Pedersen, 1972; Peters *et al.*, 1975; Peters, 1979). At one time it had been suggested that follicles grew to a certain stage of development after which their development was arrested and these would form a reserve pool of follicles (Greenwald, 1973, 1974; Schwartz *et al.*, 1974). This was based on the evidence that the ovulatory number of follicles could be increased experimentally by injection of gonadotrophins and presumably these were recruited from a reserve population of preantral follicles. This was refuted by studies using a radioactive DNA precursor and autoradiography which showed that no interruption of follicle growth could be found (Peters & Levy, 1966; Pedersen, 1972; Byskov, 1974b; Chiras, 1976).

Dynamics of follicle growth:

The pool of primordial follicles is progressively reduced throughout life (Jones & Krohn, 1961a) and in most species studied it has been shown that this loss is greatest during immature life (see Byskov review, 1987). That follicle development is not constant throughout life was concluded from studies using pulse labelling with [^{3}H] thymidine followed by autoradiography to study the kinetics of follicle growth (Pedersen, 1970a). This approach has been used by many workers (Pedersen, 1969, 1970a,b; Pedersen & Hartmann, 1971; Pedersen & Peters, 1971; Chiras, 1976; Hage *et al.*, 1978). Studies in the mouse have shown that more follicles start to grow in mice of 7 days old than in animals that are 3-5 weeks. (Pedersen, 1969) and also that follicles grow faster in these younger mice. This approach has also provided information on the timing of follicle growth in mice by observing the timing of cell divisions in autoradiography preparations (Peters & Levy, 1966) and has shown that the time taken for a primordial follicle to grow to Graafian stage is 19 days (Pedersen, 1970b).

The loss of follicles from the primordial pool must be as a result of both follicle growth and death of follicles. The distinction between these two factors is important if we are to understand why there is a greater loss during immaturity. The autoradiography approach to the study of follicle dynamics is unable to measure follicle death. Thus, to overcome many of the problems of this technique a mathematical modelling approach has been developed. The technique of compartmental modelling has been applied to measure growth ("migration") and death ("atresia") rates of subpopulations of follicles within the mouse ovary (Faddy *et al.*, 1976; Faddy *et al.*, 1983). Results obtained from this

approach have also shown changes in the rate of loss of follicles from the primordial pool with age. The results obtained from studies using this approach in mice (Faddy *et al.*, 1976; Faddy *et al.*, 1983) have indicated dramatic changes in follicle dynamics during the peripubertal period, but data at these ages has been sparse. Thus it is important to study follicular dynamics in animals with an age distribution that will be more suited to detect changes in follicle dynamics during the pre and early post pubertal age periods.

Mathematical approach to the study of follicle dynamics:

Mathematical modelling has been used to study many biological systems, and it is applied to obtain a quantitative description of a particular process in terms of a series of mathematical equations and hence to make predictions about the effects of perturbations on the system. Since mathematical models are essentially simplifying reality, they may produce a distorted representation of the system being studied, however, a good model will reveal features of a complex phenomenon which cannot be identified by other methods currently available. Mathematical modelling has been applied to studies on the ovarian system to obtain a quantitative causal physiological explanation of folliculogenesis and follicular growth processes (Read *et al.*, 1981); to quantitatively describe the control of ovulation number, (Lacker & Peskin, 1981) and to study the dynamics of follicle growth throughout life (Faddy, *et al.*, 1976; Faddy, *et al.*, 1983).

The dynamics of follicle growth is a complex process and techniques used to study it are slow yielding and laborious. The mathematical approach used to study this process involves the application of

compartmental analysis to this system (Faddy *et al.*, 1976; Faddy *et al.*, 1983). The application of compartmental modelling in this study allows us to estimate the movement of follicles with time and so obtain changes in death and growth rates. It would be hoped that with more information it would have a role in predicting changes in response to some physiological change.

Factors influencing follicle dynamics:

The role of extrinsic factors affecting the ovary, particularly gonadotrophins has been extensively studied since these hormones have been recognised for a long time and techniques are available to allow their measurement in low concentrations. Most of these studies have concentrated on larger follicles and although small follicles express Follicle Stimulating Hormone (FSH) receptors, the evidence of hypophysectomy indicates that the latter are not acutely dependent upon FSH. It is therefore assumed that intrinsic ovarian factors exist which control various aspects of follicular development. Factors present in follicular fluid have been claimed to influence the rate at which resting follicles enter their growth phase (Peters *et al.*, 1973a). Regulation of follicular maturation may also occur among the population of small growing types as indicated by mathematical modelling studies where the rate of movement out of the nongrowing pool was significantly reduced after puberty but the effect was reversed after hypophysectomy. Thus changes in rate of follicular "migration" were associated inversely with changes in the large follicle population.

The number of follicles beginning to grow each day appears to be related to the number of follicles in the pool (Jones & Krohn, 1961a;

1961b; Krohn, 1967; Krarup *et al.*, 1969). As the nongrowing pool is being depleted there is a decrease in numbers of follicles beginning to grow (Edwards *et al.*, 1977; Faddy *et al.*, 1976; Jones & Krohn, 1961a; 1961b; Peters, 1978). Artificial reduction of the non-growing pool by radiation results in a secondary reduction in the numbers of growing follicles (Peters, 1978). Results show that age dependent changes may regulate the rate of initiation or the rate of growth of small follicles. Results obtained from this mathematical modelling approach was based on an age distribution which would not allow subtle changes in the prepubertal animal to be detected. Thus how dynamics change in very young animals is still open to investigation.

Little is known of the nature and actions of intraovarian factors that are presumably involved in maintaining the orderly processes of follicular development. The interactions between and within follicle stages and their effect on follicular dynamics could be studied by applying the mathematical modelling approach. The system could be manipulated as it has been with studies on hypophysectomised animals (Faddy, *et al.* 1976). Some experimental conditions that could be tested would be 1) alteration of the ratio of large to small follicles by unilateral-ovariectomy and 2) blocking ovulation and so presumably suppressing the development of large follicles. Such experimental situations might elucidate some of the questions of intraovarian mechanisms working to influence dynamics.

<u>Scaling of ovarian parameters</u>:

Our knowledge of the ovarian system has been obtained from studies on a variety of species and so must be viewed in terms of other

limitations and pressures acting upon any particular species. Many observed differences in morphological structures may be a function of scaling and so inter specific comparisons of ovarian structure and function are important. Scaling laws or 'allometries' connecting quantities such as body weight, length and brain size have been well documented (Schmidt-Nielsen, 1984) but the reproductive system has been largely neglected (May & Rubenstein, 1984).

A number of morphological and physiological variables are scaled allometrically i.e. when two variables are plotted on logarithmic coordinates the result is a straight line. Allometric analysis has been applied to describe scaling relationships of the oocyte and follicle sizes with body weight (Parkes, 1932). Parkes looked at 7 species from 4 orders and measured the relationship between oocyte and follicle size with body weight and concluded that follicle sizes showed an allometric relationship. This type of analysis could be extended to more variables and a wider range of species in order to determine whether many of the morphological differences in the ovarian system can be explained by differences in scaling.

The general aim of the research work leading to this thesis is to observe and highlight patterns existing in follicular development and utilisation both within one particular species, namely the mouse, as well as making interspecific comparisons. The more specific aims of this thesis are to investigate some of the questions relating to the ovarian system which have been raised in this introduction.

Aims of thesis:

1) To obtain a detailed description of the follicular dynamics of young mice by application of compartmental modelling.

2) To analyse experimentally the influence of follicular interactions on the dynamics of follicle growth in two situations:

a) the premature loss of ovarian follicles and the altered ratio of large : small follicles as achieved by unilateral ovariectomy.

b) The blocking of ovulation by progesterone implants and so suppressing large pre-ovulatory follicles

3)To determine the number of precursor cells responsible for the formation of the granulosa cell layer in the mature follicle and to determine the clonal origin of the two sub-populations of granulosa cells (cumulus and mural granulosa) in the mature follicle.

4) Investigate the incidence of polyovular follicles in several species and undertake a detailed investigation of a particular species in which they are particularly abundant (domestic bitch).

5) To determine whether a scaling (allometric) relationship exists for a range of ovarian parameters across several species.

Chapter Two:

Follicular Kinetics in the ovaries of CBA/Ca mice from birth to 98 days

Introduction

The purpose of this study was to construct a mathematical model which would describe the dynamics of follicular growth and death in the ovaries of CBA/Ca mice. The mammalian ovary presents an interesting system for the study of cell dynamics, since the stock of oocytes is formed and fixed by birth or shortly afterwards. During the lifespan this stock of follicles becomes progressively depleted through death and recruitment towards ovulation.

Lipschutz in 1927 proposed a theoretical law of follicular constancy to explain the relationship between the number of oocytes shed at oestrus and the total number of ovarian oocytes available. Following from this there have been several attempts to describe the dynamics of ovarian follicle utilisation which have applied a number of methods. These include the estimation of the rate of increase of granulosa cells either by counting mitotic indices (Bullough, 1942; Zuckerman, 1951). Such methods could only provide a rather rough approximation to the turnover of the follicular population.

A crucial problem is to have a technique which is amenable to study the utilisation of follicles throughout life. This has been attempted in mice using two methods.

1) Labelling with tritiated thymidine

2) Mathematical models.

The "marking" of cells with radioactively labelled thymidine with autoradiographic detection at different time intervals was a significant advance which enabled workers to visualise the dynamic processes of follicular utilisation, (Peters & Levy, 1966; Pedersen, 1969, 1970a,b; Pedersen & Hartmann, 1971; Pedersen & Peters 1971; Hage *et al.*, 1978). However, this method is unable to identify the role played by follicular death and is restricted by practical problems in large animals by the need to inject sufficient radioisotope.

The mathematical approach to the study of follicle dynamics has been used to compare the follicle dynamics in several strains of mice in both natural and experimental conditions, e.g. hypophysectomy and in hypogonadal mutant mice (Faddy *et al.*, 1983; Halpin *et al.*, 1986). The development of mathematical models in the study of follicular dynamics allow for death rates to be included and could be applied to animals that are not suitable for radioisotope studies, if histological material was available. In some of these studies (Faddy *et al.*, 1983) the age span of the mice ranged from birth to 600 days old, but most observations were at the upper end of this age range.

The present study was designed to examine follicle dynamics in detail from birth through puberty to reproductive maturity, and to determine whether changes in the dynamics of small follicles corresponded to major transitions in ovarian function and its hormonal regulation. Mathematical modelling has been employed in this study as this has been established as a useful tool for analysing this problem.

When a mathematical approach is taken the biological system being studied must be carefully considered. It is important to appreciate the assumptions inherent in the chosen method of analysis and that these can be met. In the present case the branch of biomathematical modelling known as compartmental analysis has been chosen as this is most appropriate when studying the time dependent movement of follicles from stage to stage and their loss by death. Some of the underlying principles and assumptions involved will now be discussed.

The object of the mathematical modelling approach to the study of follicular dynamics can be twofold:

1) To obtain a description of a particular process in terms of a series of mathematical equations.

2) To use this in a predictive way.

The particular type of model employed in this study allows us estimate the movement of follicles with time and so obtain changes in death and growth rates. It would be hoped that with more information it would have a role in predicting changes in response to some physiological change.

Compartmental analysis:

A trend towards a greater emphasis on the quantitative treatment of data developed in the biological sciences around the 1940's. There was an increasing use of differential equations to obtain a mathematical representation for observed phenomenon, particularly dynamic phenomena and the study of the behaviour of tracer molecules.

The term compartment was first used in this context by Sheppard (1948) and the term has become interchangeable with pool and has been defined by Atkins (1969) as "a quantity of a substance which has a uniform and distinguishable kinetics of transformation or transport". Compartmental systems are represented diagramatically using a circle to represent a compartment and arrows to represent the transfer of material in and out of compartments.

The definition of compartmental analysis is given by Godfrey (1983) as "models consisting of a finite number of homogeneous, well mixed subsystems called compartments which exchange with each other and the environment so that the quantity or concentration of material within each compartment may be described by a first order differential equation." This approach can be used to model the kinetics of substances or material through discrete stages if the following assumptions are met:

1) There is a fixed source of material
2) compartments occupy different spaces
3) There is a uni-directional flow of material between compartments.
4) All compartments are interdependent.

A compartmental system can be used to model the kinetics of one substance and in this case the compartments occupy different spaces and the inter-compartment transfers represent the flow of material from one location to another.

In areas such as pharmocodynamics the kinetics of more than one substance is of interest, for example, the kinetics of a drug and its metabolites and in this case different compartments may occupy the same space and some of the inter-compartmental transfers represent transformation of one substance to another.

The most general form of compartmental equations (Sandberg, 1978) for a system with p compartments is:

$$dx_i = f_{i0} + \Sigma f_{ij} - \Sigma f_{ji} - f_{0i}, \quad i = 1,2,.....,p.$$

where x_i is the amount of material in compartment i

An early application of a simple compartmental analysis was published in 1943 (Zilversmit *et al.*, 1943), before the term compartment was introduced by Sheppard in 1948. Workers in the early days of compartmental analysis assumed that the behaviour of the population being studied was strictly deterministic (Zilversmit, 1943; Sheppard & Housholder, 1951). These assumptions limit the applicability of these models to a few systems; consequently, stochastic compartmental analysis was developed to handle assumptions of probabilistic behaviour of components, (Bartlett, 1949). Workers involved in compartmental modelling (Cornfield *et al.*, 1960) concluded that the stochastic type model was more realistic. This type of analysis assumed that observations were independent over time (Matis & Hartly, 1971) and would be inappropriate to study dynamics of a population over time.

Stochastic compartmental model was extended by Faddy (1976) to consider time dependence. The model of Faddy (1976) was applied to the study of ovarian follicle dynamics. This model is formulated stochastica -lly and describes the growth of follicles in terms of five stages of development and so there are five corresponding compartments (Faddy *et al.*, 1976). This model has been used to study follicular dynamics in hypophysectomised mice and of four strains of mice (Faddy *et al.*, 1983) and of the mutant strain of hypogonadal mice (Halpin *et al.*, 1986).

The application of compartmental modelling employed in this study allows us to estimate the movement of follicles with time and so obtain changes in death and growth rates. It would be hoped that with more information it would have a role in predicting changes in response to some physiological change.

Materials and Methods:

Animals:

The mice used in this study were from the inbred CBA/Ca strain reared in the Faculty Animal Area of the University of Edinburgh. The animals were housed in thermostatically controlled rooms at 21°C with controlled lighting conditions under a photo-period of 14 hours beginning at 0700h GMT and provided with a pelleted diet with water ad libitum. All breeding pairs were checked daily for litters and the day of delivery was noted and recorded as day 1 of life. To reduce any possible external sources of variation only those animals from litters of a size ranging from 5-10 pups were included in this study. This was

done in order to minimise differences in weight of pups which may affect follicular development.

Since other studies on follicular dynamics have concentrated on adult animals this study was designed to look in detail at younger animals. Animals within an age range of 1-100 days were selected with almost 50% of these being less than 25 days old. Animals from all age groups were selected at random. Prior to weaning none of the litters were split, thus standard litter sizes and conditions were maintained at all stages. All animals were weighed and examined for vaginal opening prior to being killed. Daily vaginal smears were taken by lavage from those animals in which cycle activity had been established, and only those showing a pro-oestrous smear were selected. Thus the stage of cycle was standardised in mature animals, this being important since the numbers of large follicles present are influenced by hormonal fluctuations of the oestrous cycle (Mandl & Zuckerman, 1952; Pedersen, 1970, 1972).

Collection of Ovaries:

All animals were killed between 14.30 and 15.30h. G.M.T. since a circadian rhythm may exist in the follicle population profile (Sahu, 1985). Animals up to 20 days of age were killed by decapitation and those over 20 days by cervical dislocation. The ovaries were excised from the animal and the surrounding fat dissected free and fixed in aqueous Bouin's fluid for twelve hours. The ovaries were dehydrated through graded alcohols and cleared in cedar-wood oil. To ensure maximum clearing the ovaries were placed in toluene for one hour prior to wax impregnation under vacuum. The ovaries were embedded in

paraffin wax (Paraplast, Lancer, St Louis, U.S.A.) in pairs. Each paraffin wax block was serially sectioned at 7μm, stained with haematoxylin and eosin and mounted with DPX.

Classification of follicles:

In all species there is a development of follicles from small single-layered stages to larger multilayered antral follicles capable of being ovulated. Follicular growth is a continuous process but in order to measure it, it is necessary to classify follicles at discrete stages along this continuum. There are two main systems for the classification of follicles, namely, those of Mandl and Zuckerman (1951) and Pedersen and Peters (1968). Other workers have adopted the diameters of follicles as a method of classification especially for rat and hamster ovaries. In this study the stages of follicular development were classified according to the criteria of Mandl and Zuckerman (1951). This system is based on the number and appearance of the granulosa cell layers surrounding the oocyte and follicles are classified as follows:

stage I Oocyte surrounded by a single layer of squamous granulosa cells.

stage II Growing oocyte surrounded by a single layer of cuboidal granulosa cells.

stage III Growing oocyte surrounded by two layers of cuboidal granulosa cells.

stage IV Growing oocyte surrounded by three layers of granulosa cells.

stage V Oocyte surrounded by four or more layers of granulosa cells but no antrum.

stage VIa Antrum forming

stage VIb Oocyte surrounded by cumulus mass and a fully formed antrum

This classification system is illustrated in figure 2.1.

Differential counts of follicles:

In this type of study ideally the best way to obtain the information on numbers of follicles would be to look at every section, however, this method can also lead to overcounting and the logistics of counting every section is a problem. What is required is that a compromise is reached between data accuracy and effort required to generate it. If every section is counted then the limitations imposed by time results in the minimum number of whole ovaries being examined. The object of this study was to balance the accuracy of the data with maximum number of individuals in order to obtain longitudinal information. The best compromise is therefore to choose a sampling frequency.

Follicles classified as stages I-IV were counted in every tenth section. This sampling frequency was decided upon after a preliminary study of different sampling points at every 5^{th}, 10^{th} and 20^{th} section revealed that it gave satisfactory precision within a realistic timespan for examining many ovaries.

No significant differences were found between sampling every fifth and every tenth section but significant differences were found at all three stages between counts from every tenth section and every twentieth section (see figure 2.2).

Figure 2.1:
Classification system of follicles based on the number of granulosa cell layers (after Mandl & Zuckerman, 1951).

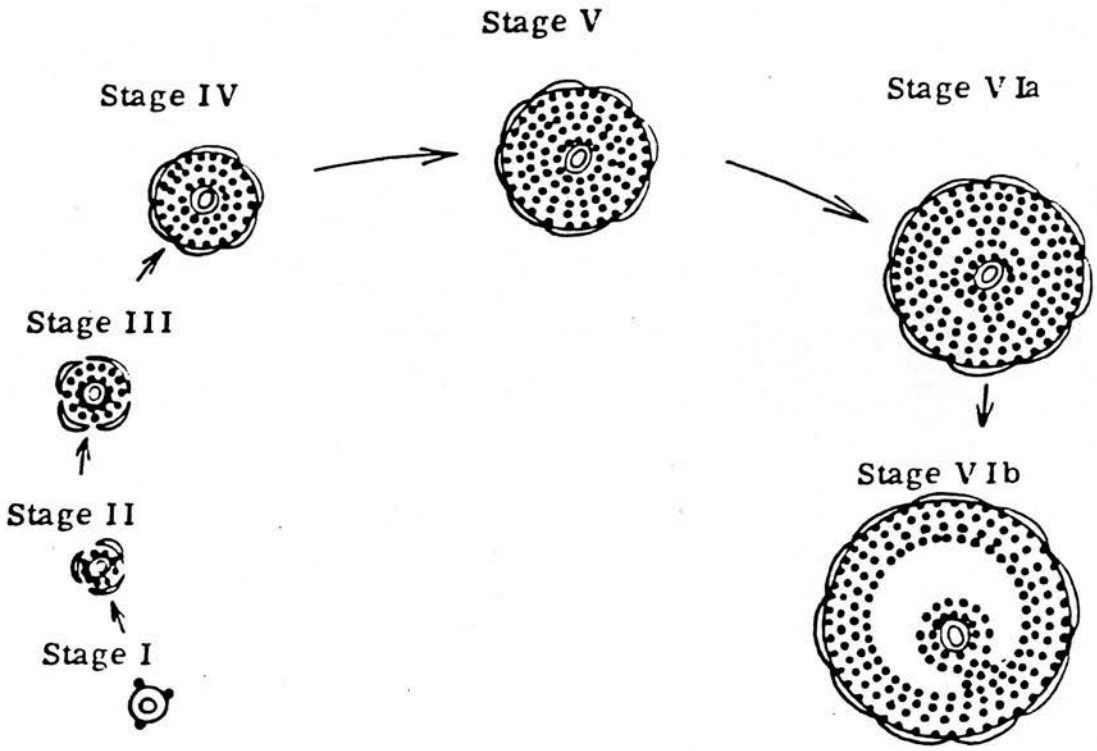
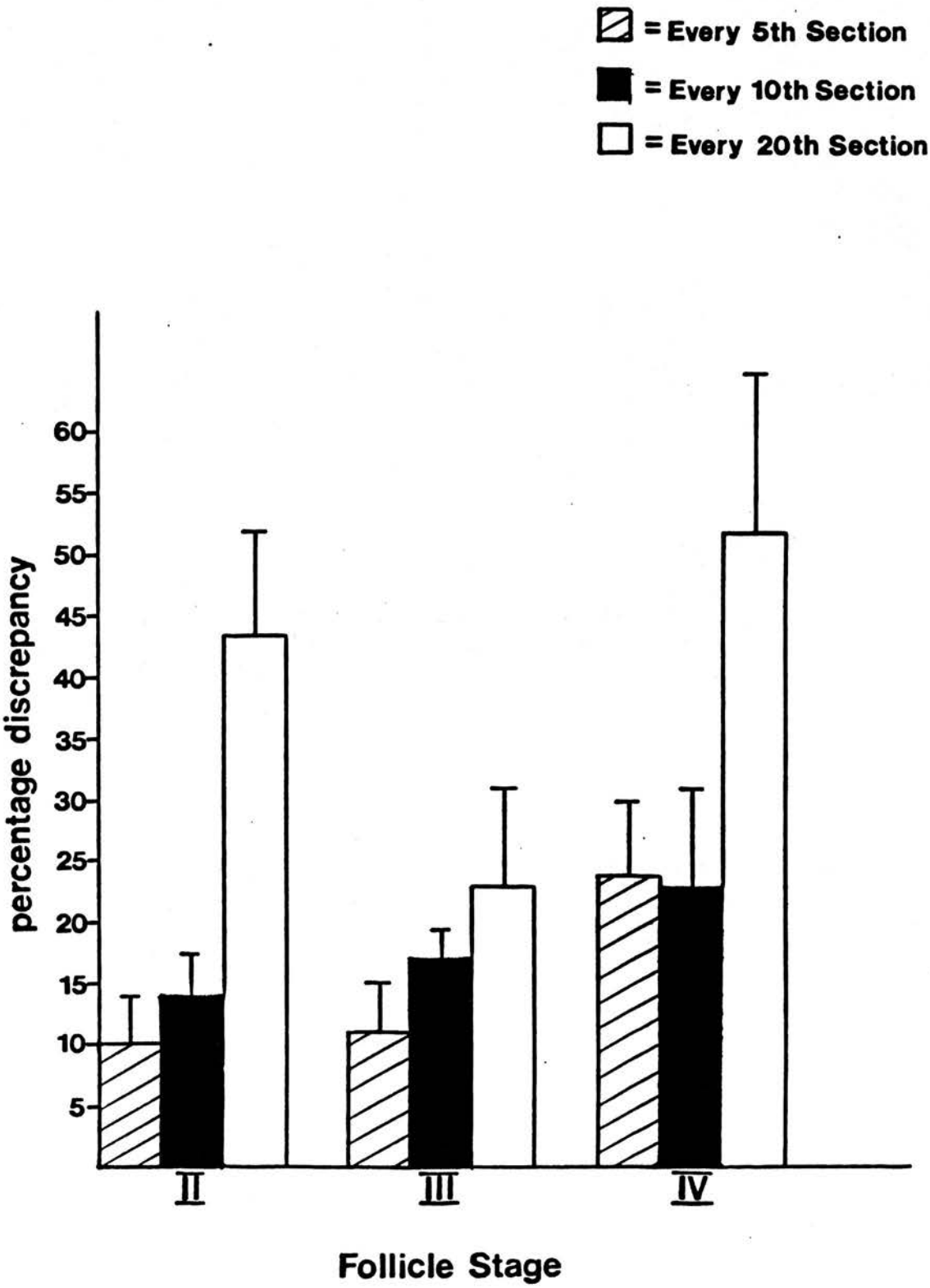


Figure 2.2:
Mean discrepancy (±S.E.M) between counts of follicles from every section and those obtained at a sampling frequency of every, 5th, 10th and 20th section.



The nucleolus was used as a marker for follicle counting, the nucleolar diameter was measured since it varied according to the stage of follicular development.

Every section was examined to obtain estimates of follicle stages V, VIa and VIb since these are less abundant.

To determine the reproducibility of these counts follicles in one immature and one adult pair of ovaries were counted on six separate occasions and the coefficient of variation calculated.

Correction factors:

Appropriate correction factors were calculated to compensate for overcounting (Abercrombie, 1946; Zuckerman, 1951) from the equation:

$$\frac{\text{Section thickness}}{\text{Section thickness + nucleolar diameter}}$$

To obtain the total numbers of follicle stages I-IV the values were multiplied by the sampling frequency (10) and by the correction factor.

Atresia of follicles:

Follicles were examined for signs of degeneration. This was most difficult to determine in follicles at early stages of follicular development. The largest cross sections of stages V, VIa and VIb were examined for signs of atresia. The criteria used were as follows:

Atresia, type1: Presence of 3-20 pyknotic granulosa cells.

Atresia, type2: Presence of more than 20 pyknotic cells in the same section and/or obvious degeneration of the oocyte or its membranes.

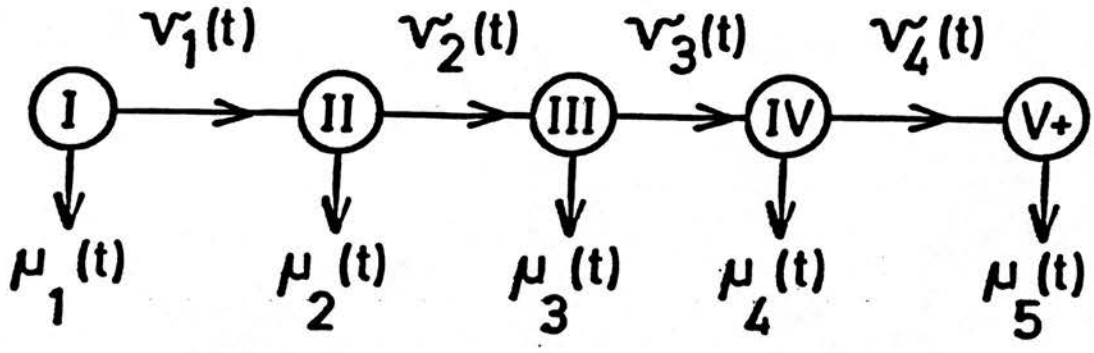
Mathematical modelling:

The data obtained by differential counts of follicles over an age span of 1-98 days were analysed by mathematical modelling. Before applying any form of mathematical analysis to a biological system, any assumptions and limitations must be carefully considered before an appropriate analysis can be chosen. In this study the biological system of interest, viz the ovarian follicular population, is highly suitable for the application of a compartmental analysis. Since the follicular system is a closed one with follicles leaving the primordial 'pool' either by death or by growth to the next stage of development, and since discrete stages of development can be identified it seems an appropriate system to apply compartmental analysis.

The data were analysed using the compartmental model of Faddy, (Faddy *et al.*, 1976; Faddy *et al.*, 1983; Halpin *et al.*, 1986). The model describes the growth of follicles through and death from five stages (groups I-V+) which are represented by five corresponding compartments (see figure 2.3). The assumptions of the model as applied to these data are:

1) Follicles migrate from the reserve population of follicles (stage I) into stage II and so on through the stages until the follicle attains preovulatory ripeness at the terminal stage V+.

Figure 2.3:
Diagramatic representation of compartmental (or "death/migration") model for ovarian follicle dynamics.



The time dependent migration rates describing the growth of follicles from group to group at varying age (t) throughout the lifespan are represented by: $\nu_1(t)$, $\nu_2(t)$, $\nu_3(t)$ and $\nu_4(t)$. The death rates for each group are represented by: $\mu_1(t)$, $\mu_2(t)$, $\mu_3(t)$ and $\mu_4(t)$.

2) The numbers of follicles in each of the four growing stages are interdependent as they are all influenced by the preceding stages and in turn influence stages that follow.

3) The rates of migration and death are age dependent.

With this particular application of compartmental modelling the model has five compartments. The mean compartment size at any given time point can be found by the solution of differential equations :

Equation:

$$\frac{d\,\lambda i\ t}{dt} = \nu_{i-1}(t)\,\lambda_{i-1}(t) - [\nu_i(t) + \mu_i(t)]\lambda_i(t)$$

rate of change of compartment i = flow into i from i-1 − flow out of i

This equation represents the mean rate of change in numbers of follicles at any given stage (compartment) thus from this curves can be constructed and rates of change. If the mean number of follicles at given stages at a range of known ages are estimated then by applying the data to the mathematical model the parameters can be computed. The parameters describing the model are:

1) Mean size of group I (λ).

2) The migration rates of follicles from stage I-II ($\nu_1(t)$).
The migration rates of follicles from stage II-III ($\nu_2(t)$).
The migration rates of follicles from stage III-IV ($\nu_3(t)$).
The migration rates of follicles from stage IV-V+ ($\nu_4(t)$).

3) Death rates from I ($\mu_1(t)$).

Death rates from II ($\mu_2(t)$).

Death rates from III ($\mu_3(t)$).

Death rates from IV ($\mu_4(t)$).

Death rates from V ($\mu_5(t)$).

In this application of compartmental modelling the system has five compartments. Each of these compartments represent a stage of follicular development, stages V, VIa and VIb were amalgamated as one group, V+, as there are few follicles in these groups and so sampling may lead to serious errors in estimating parameters.

These migration rates describe the growth of follicles from group to group at varying age (t) and also the death of follicles from each of these stages. The rates estimated have a simple probabalistic interpretation e.g. For, $\nu_1(t)$ and $\mu_1(t)$: A follicle in group I at age t has become a group II follicle by age $t+\delta t$ with probabilty $\nu_1(t)\delta t$. A follicle in group I at time t has died by age $t+\delta t$ with probability $\mu_1(t)\delta t$.

Non-Parametric Regression:

In this study non-parametric regression techniques were used to aid the fitting of the parametric model. This is a technique which does not assume any particular mathematical distribution. For each of the five groups a non-parametric estimate of the curve describing the dependence of follicle counts on age was obtained using a spline smoothing approach (Silverman 1985). This non-parametric regression gives an impression of overall changes in pattern is obtained and

indicatations if transitions in growth and death rates are occurring. The rates of migration are not constant throughout life and so estimates from the non-parametric techniques determine where age dependency in the migration may be occurring and thus appropriate age dependency can be incorporated into the model.

Results:

Figure 2.4(a-e) shows the results of follicle counts at each stage of development with age. The data is characteristically variable. Each graph has two curves, one represented by a broken line which is the age-dependent curve determined from these data by non-parametric regression techniques (Silverman, 1985). These graphs show the triphasic nature of the non-parametric regression curves in stages II, III & IV with troughs occurring at around 20 and 60 days of age. The continuous line represents the parametric curve obtained by fitting the data to the parametric compartmental model.

The non-parametric regressions identify three distinct age phases: (1) 0-20 days, (2) 20-60 days and (3) 60-100 days, with the 20 and 60 day transition points corresponding to troughs in group II, III and IV curves. From this it was clear that some form of age-dependence in the growth and death rates of follicles would have to be incorporated into the model. The parametric compartmental model described by Faddy (1976) was then fitted to the data and age-dependence was incorporated into this. Thus, rates of migration and death of follicles were not constant throughout life, however, rates within these age groups have been assumed to be constant.

Figure 2.4 a-e:

Graphs a-e (pages 33 & 34) represent the number of follicles (stages I-V+) in 95 virgin CBA/Ca mice ranging from 1-98 days post partum. The data were obtained by differential counting of the follicle stages. The continuous line shows the fit of the parametric curves obtained from the application of compartmental modelling to the data. The interrupted line corresponds to the non-parametric regressions. Where there are less than 95 points a value is present more than once.

Fig. 2.4a

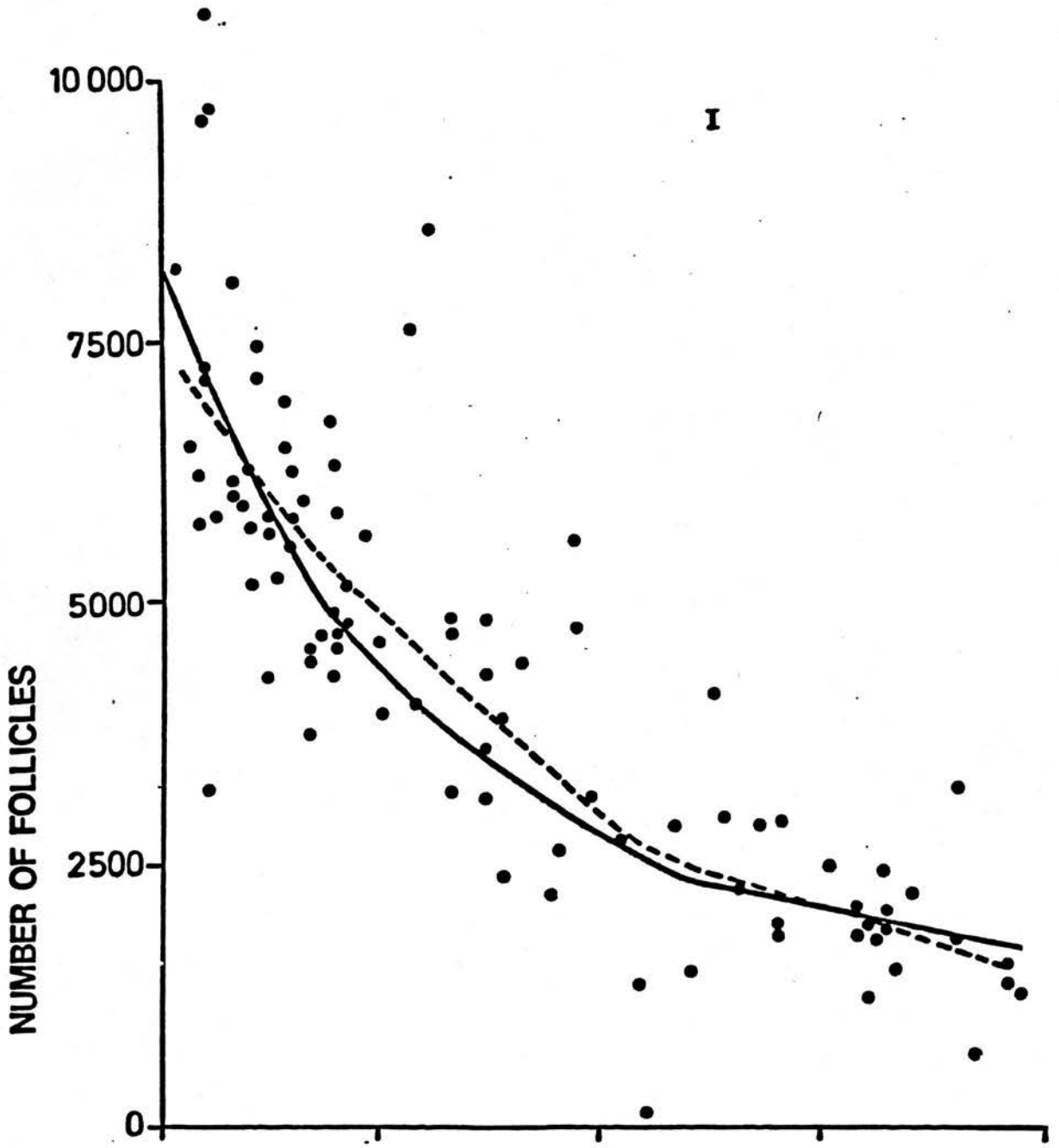


Fig. 2.4b

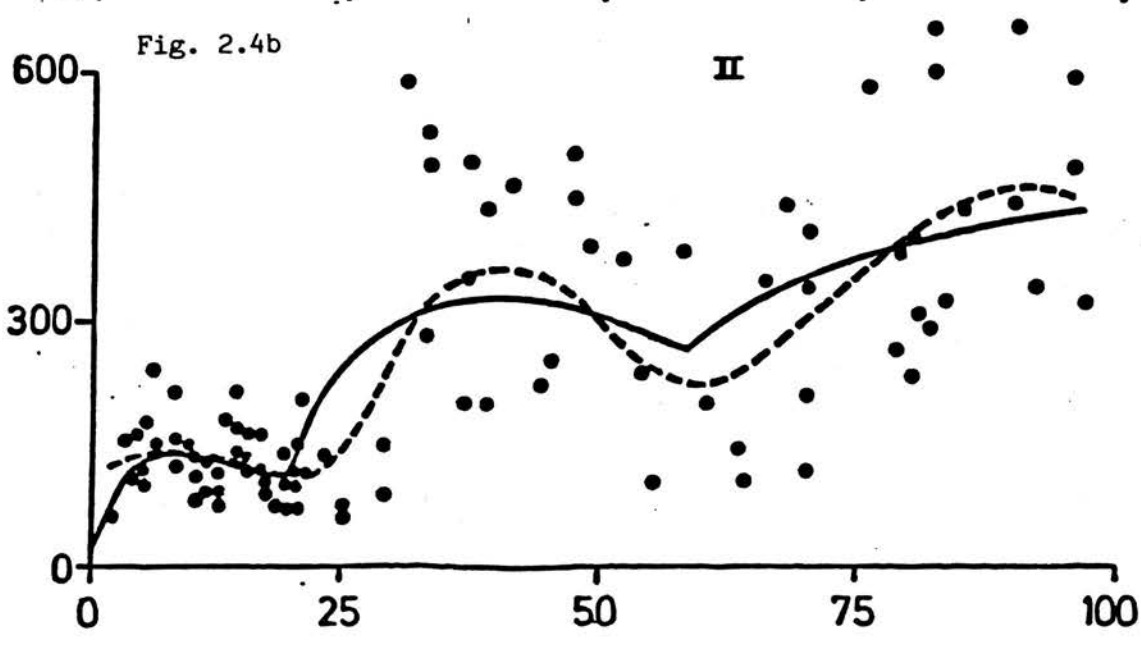


Fig. 2.4c

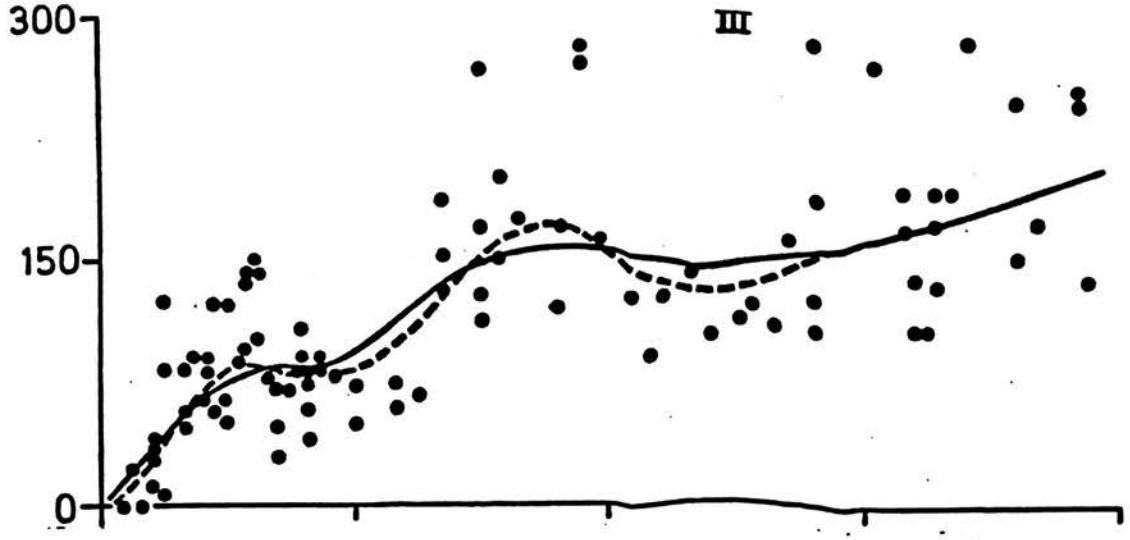


Fig. 2.4d

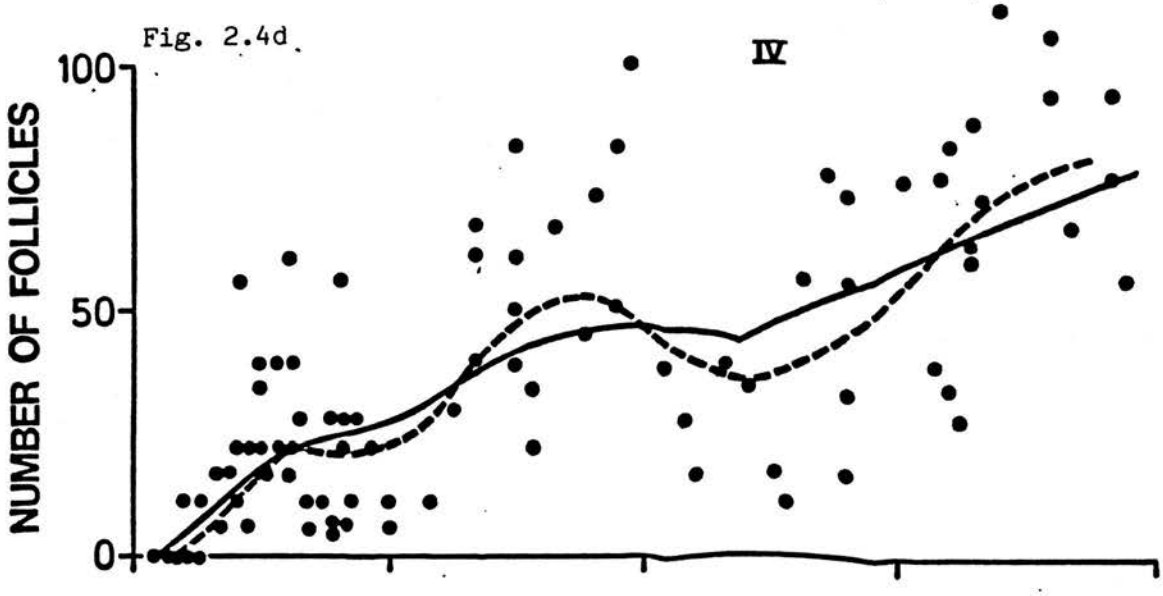
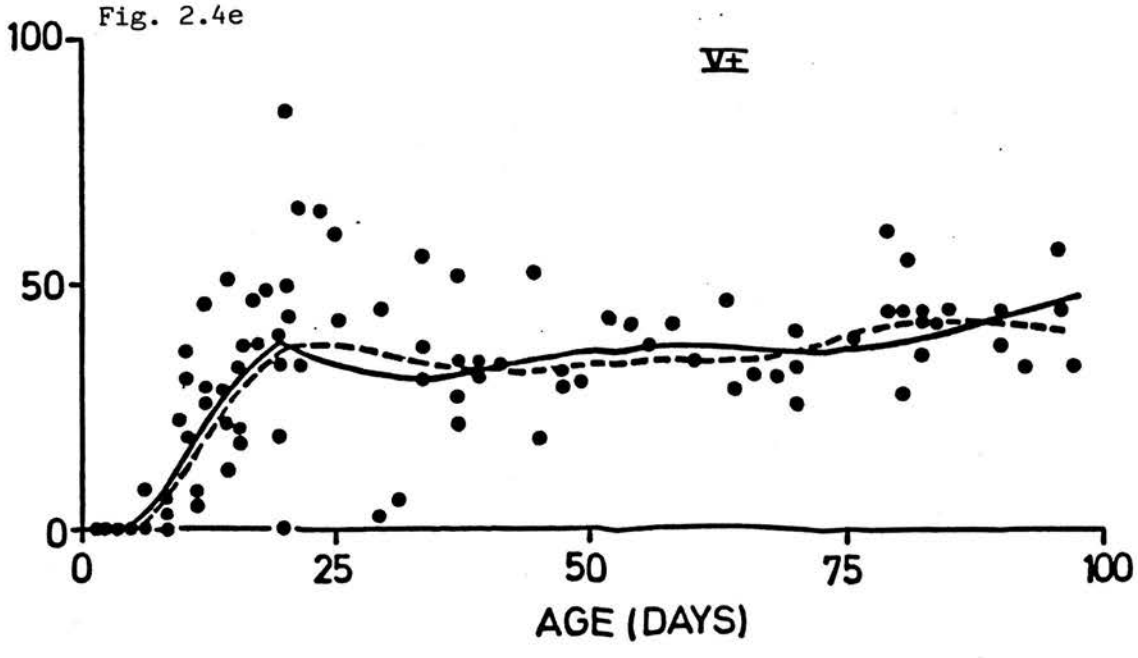


Fig. 2.4e



The parametric curves giving the mean numbers of follicles in all five stages are shown by the continuous line on figure 2.4. The parametric curves are specified by three stage step function forms for the compartmental transition rates and these closely correspond to the triphasic non-parametric regression curve. From these curves three estimates for each growth and death rate corresponding to the age phases were obtained.

Significance should not necessarily be attached to the three phases nor the ages at which the transitions occur. These phases are in a sense arbitrary devices used to represent a complex process in a tractable way with the transitions representing the dominant changes that occur in follicle growth and death rates.

Follicle counts were found to be very reproducible (Fig. 2.5). The repeat counts generated coefficients of variation ranging from 3-7%.

The estimates of the parameters of the model are shown in figure 2.6. In all there are 28 parameters, 9 at each age phase and one for the estimation of total numbers of follicles at birth.

It is an assumption of the model that the stock of follicles is fixed at birth and on this basis the model predicts that the follicular pool has a mean size of 8177+/- 642. The results of the modelling will be examined by looking at each phase in turn.

Figure 2.5:

Reproducibility of follicle counting: Each point represents a repeat count for four stages of follicular development obtained by counting follicles from the ovaries of 2 animals six times. The coefficient of variation ranges from 3-7%.

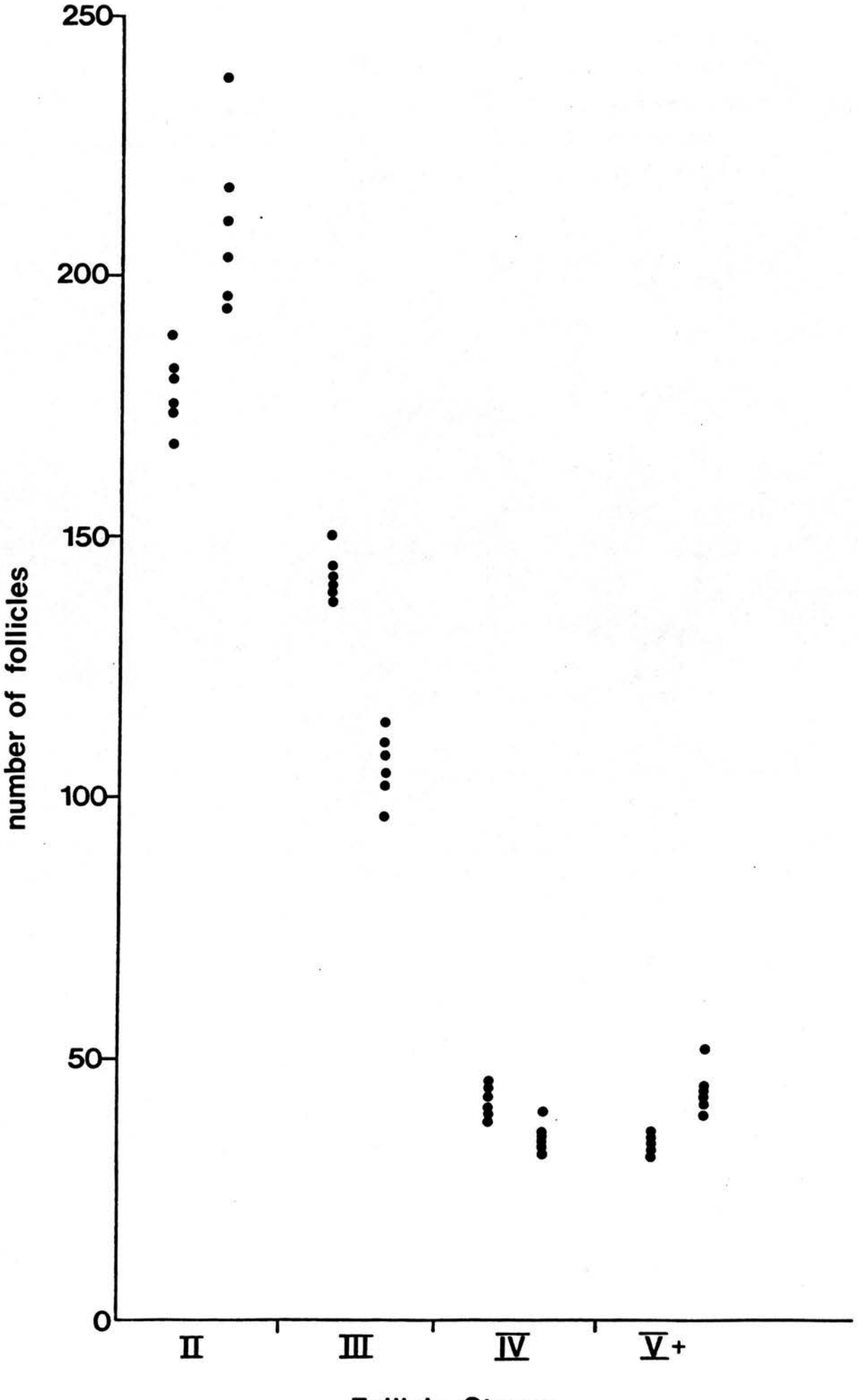
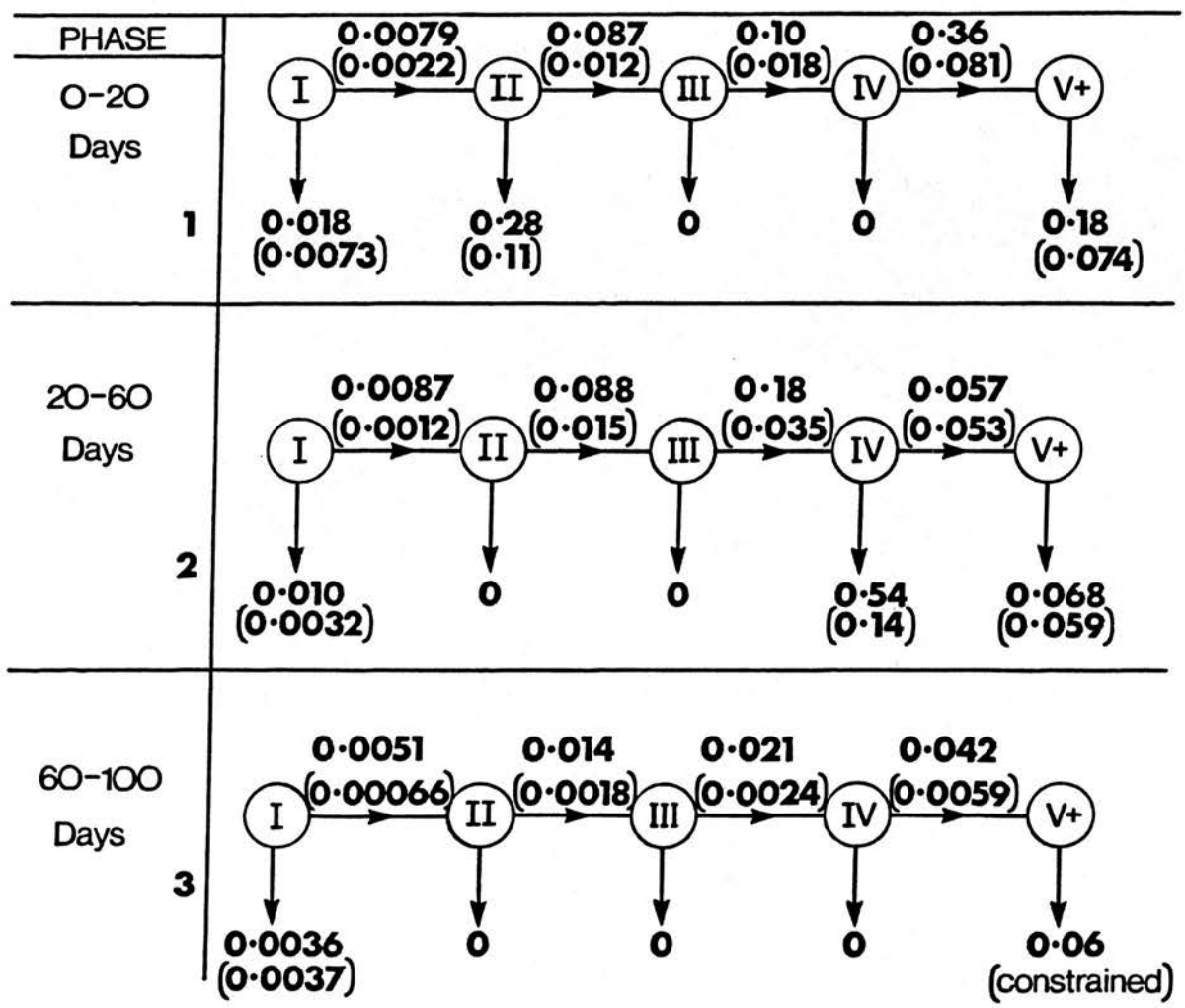

250
200
150
100
50
0
number of follicles
II
III
IV
V+
Follicle Stages

Figure 2.6:

This figure shows the parameter estimates for growth (migration) and death (atresia) through five stages of follicle development, three age phases are indicated. Standard errors of these estimates are shown (in parentheses).



Phase One:

This can be considered as an immature phase with it being identified as spanning from age 0-20 days. The numbers of primordial follicles decline rapidly with this being a result of deaths at this stage and growth to the next stage of development (stage II).

During phase one it is estimated that over 2/3 of the losses from the follicular pool (stage I) are because of deaths with less than 1/3 growing to the next stage. Once a follicle starts to grow the rates of movement through successive stages i.e. from II-III, III-IV and from IV-V+ is rapid without significant numbers of deaths at stages III or IV. However, all follicles must degenerate at the stage V+ stage of development since at these ages ovulation does not occur.

Table 2.1 gives values of average numbers of follicles expected to leave the compartments per day, estimated from the mean numbers shown in figure. On the day of birth there are zero follicles at stages III, IV & V+. It would seem that follicles enter their growth phase shortly afterwards as follicles at stage II of development are present by day 2. Stage V follicles are present in most animals by 9 days of age.

Phase Two:

This phase has been identified as occurring between 20 - 60 days of age and may be considered as an intermediate phase between immaturity and maturity. At the onset of this phase the numbers of stage I follicles have been drastically reduced with these numbering approximately 5000 in the ovaries of 20 day old animals, which is less than 60% of the original numbers present at birth.

Table 2.1:
Average numbers of follicles leaving the stages (compartments) per day, either by growth to the next stage or death. The divisions correspond to the three age phases identified by the model.

Age	I→	→II	II→	→III	III→	→IV	IV→	→V→	V→
0	210	65	0	0	0	0	0	0	0
7	180	54	52	12	4,8	4,8	3,0	3,0	1,0
14	150	45	47	11	8,3	8,3	7,3	7,3	4,4
21	89	41	12	12	16	16	15	1,4	2,6
28	78	36	25	25	19	19	17	1,7	2,2
35	68	32	29	29	25	25	24	2,3	2,1
42	60	28	29	29	28	28	27	2,6	2,2
49	52	24	28	28	29	29	28	2,7	2,4
56	46	21	25	25	27	27	27	2,6	2,5
63	19	11	4,1	4,1	3,1	3,1	2,0	2,0	2,2
70	18	11	4,8	4,8	3,3	3,3	2,3	2,3	2,3
77	17	10	5,3	5,3	3,5	3,5	2,5	2,5	2,3
84	16	9,4	5,7	5,7	3,8	3,8	2,8	2,8	2,4
91	15	8,8	6,0	6,0	4,1	4,1	3,1	3,1	2,6
98	14	8,3	6,3	6,3	4,3	4,3	3,4	3,4	2,8

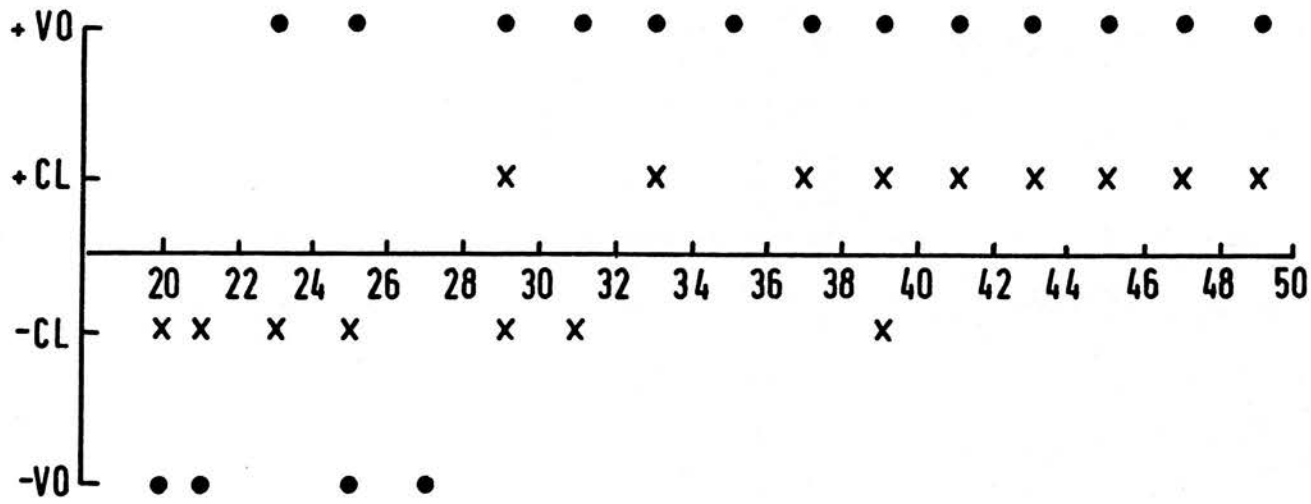
Figure 2.7:

Emergence of Vaginal opening (VO) and Corpora lutea (CL) in young CBA/Ca mice.

This figure represents the age in days that vaginal opening and corpora lutea were first found

+VO/-VO indicates vaginal opening present/absent.

+CL/-CL indicates presence/absence of corpora lutea


+VO
+CL
-CL
-VO
20
22
24
26
28
30
32
34
36
38
40
42
44
46
48
50

During this age phase a decline in the utilisation of stage I follicles was observed. This decline was as a result of less deaths at stage I as the growth rates at phases 1 and 2 were similar.

During this phase sexual maturity was attained, vaginal opening began to occur at day 23 and by day 28 all animals examined had vaginal openings. Corpora lutea first appeared at 29 days of age (see Figure 2.7). There was no evidence of follicles dying at stages II and III, however there was evidence of follicle death at stage IV (see Figure 2.6 of parameter estimates for the second phase). The rate of movement from I-II and from II-III was similar in both phase 1 and 2 , however there are differences in the rate of movement from III-IV, but with the indication of death at stage IV this results in a slower rate of movement from stage IV - V.

Phase Three:

This phase may be regarded as a mature phase, since by this time all animals were cycling. At the beginning of this phase (60 days), the number of stage I follicles was reduced to around 30% of the number present at birth. During this phase a significant decrease in deaths from the pool of stage I follicles was indicated and for the first time the number of follicles starting to grow exceeded those dying. In this final phase covered by the model no deaths of intermediate stage follicles were indicated.

Table 2.1 shows the average number of follicles leaving from one stage and entering another per day at various ages. This shows that the number of follicles leaving the pool of follicles (stage I) decreases

from 210 per day at the onset of follicular growth (phase 1) to 89 per day at the onset of phase 2 (age 21 days) until the onset of phase 3 where this figure has been reduced to 19.

Atresia of follicles:

Atresia was scored for in stage V+ follicles which were subdivided into three stages (large multilayered follicle), VIa (antral follicle), VIb (antral follicle). Figure 2.8 shows the number of each of these stages at present at the three age groups. The results show that there is a changing profile in the distribution of numbers at these stages with age. There are significantly less follicles at stage VIb in ovaries of animals aged from birth to 20 days but there is no significant difference in this distribution between animals in the other two groups.

The data obtained from observations on atresia are shown in figure 2.9. Atresia was scored in the V+ group only as degenerating follicles at earlier stages of follicular development could not be identified morphologically. Figure 2.9 shows the percentage of follicles at stage V, VIa and VIb that show signs of atresia, for the three age groups. Similar levels of atresia at stages V and VIa were observed in ovaries of animals from birth to 20 days of age with a significant increase to 45% atresia in the stage VIb population of follicles compared with 18% and 13% at this stage during phases 2 and 3, respectively. In both groups of animals at phase 2 and 3, the percentage atretic follicles peak at follicle stage VIa with this value being 25% during phase 2 and 33% during phase 3.

Figure 2.8:

Mean number (+S.E.M) of follicles at stages V, VIa & VIb in three age groups.

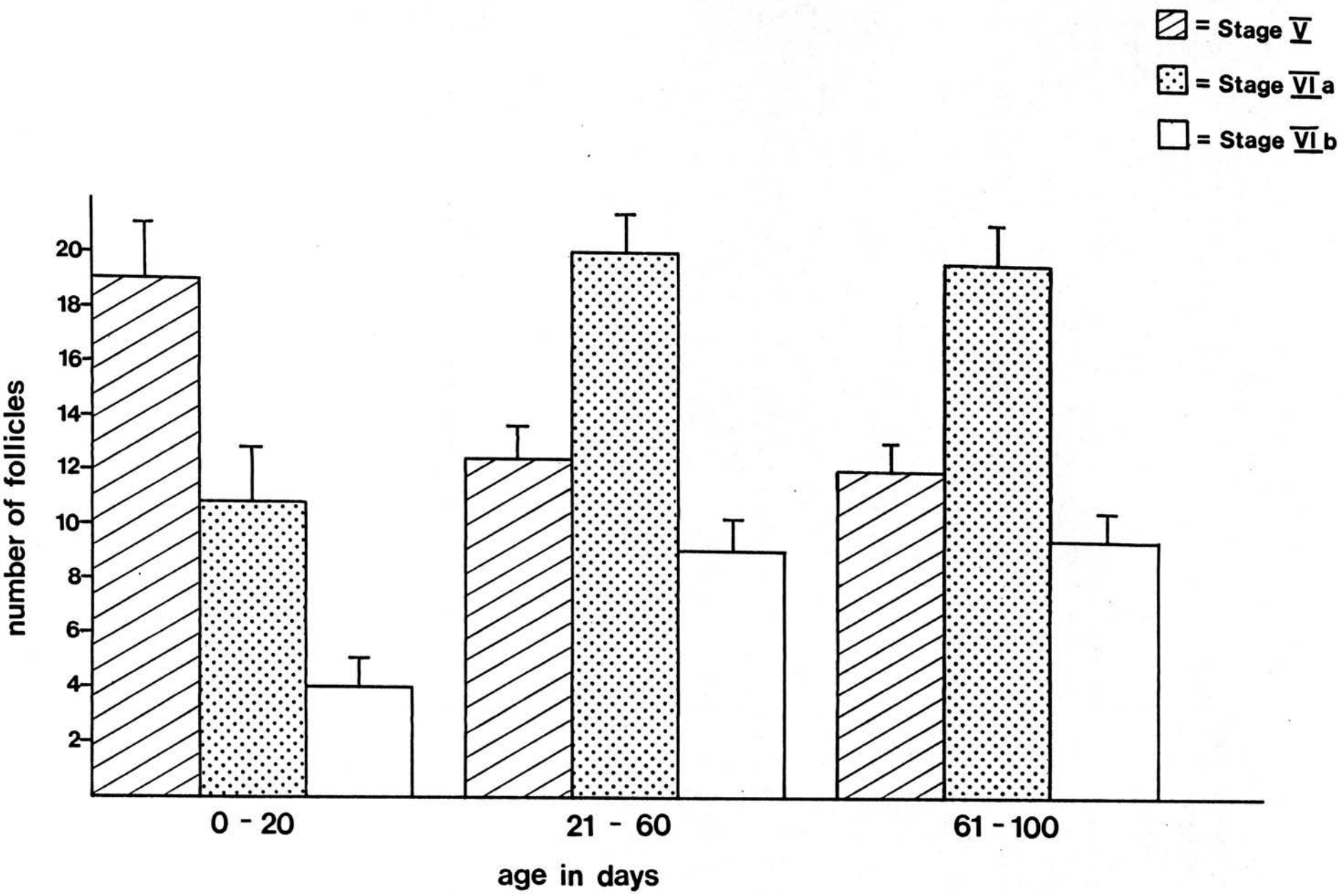
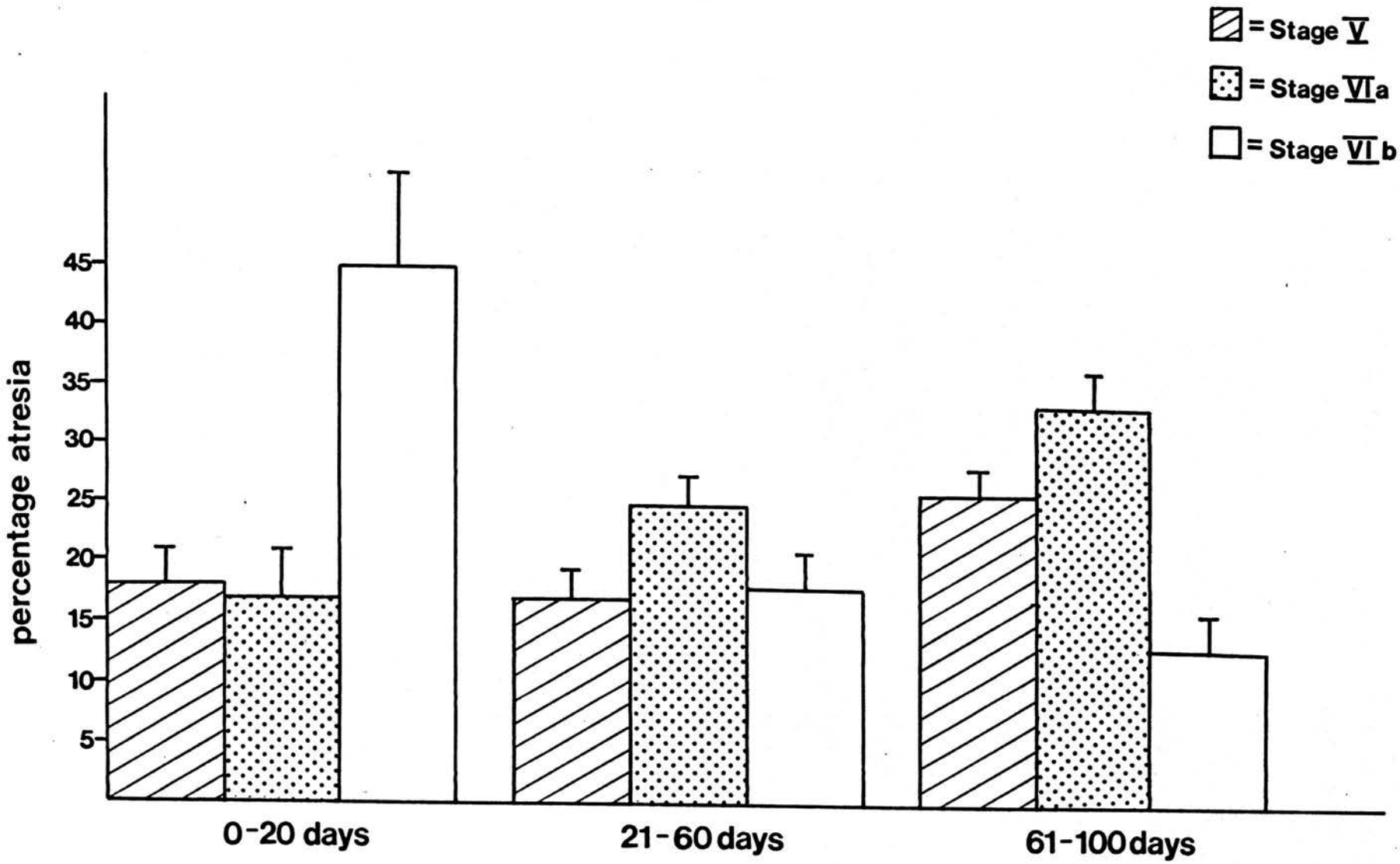

= Stage V
= Stage VI a
= Stage VI b
number of follicles
20
18
16
14
12
10
8
6
4
2
0 - 20
21 - 60
61 - 100
age in days

Figure 2.9:

Mean percentage (+S.E.M) of follicles at stages V, VIa & VIb in three age groups showing signs of atresia.


= Stage V
= Stage VIa
= Stage VIb
percentage atresia
45
40
35
30
25
20
15
10
5
0-20 days
21-60 days
61-100 days

Discussion:

The stochastic compartmental model has been used previously to estimate the kinetics of follicle development in various strains of mice (Faddy *et al.*, 1976; Faddy *et al.*, 1983). The earlier studies involved the use of data which had not been collected with mathematical modelling in mind and the stage of the oestrous cycle was not controlled. This present study is the first one which has been designed to follow detailed temporal changes in follicle utilisation from birth to maturity in animals kept under strictly controlled environmental and physiological conditions. The major feature of this new model is the greater degree of complexity inferred during ovarian maturation compared with earlier models. This is expressed by the 3 age phases during the first 100 days of life as opposed to 2 phases observed by Faddy *et al.*, (1983). This study is also novel in that it involved the use of non-parametric regression techniques which aided the fitting of the parametric model for follicle dynamics.

This study was carried out under very precise conditions in order to reduce possible sources of external environmental variation and physiological and genetic variation. Despite these precautions there was a surprising amount of variation in the data. As it was possible that this variation could have arisen as a result of imprecise counting the counting techniques were evaluated more closely by testing for repeatability. The repeat counts indicated a satisfactory narrow range of values as indicated by the coefficient of variation. Hence, it is concluded that most of the variation present is a structural feature of the ovarian follicular population although this could be influenced by pre-natal factors operating differentially in the uterus.

This particular model allows us to predict the movement of follicles with time and to obtain changes in death and growth rates through development. The model derived from the analysis of the data indicates a degree of complexity in follicular dynamics during life. As an aid to understanding the complexity of the system 3 age phases have been identified. Although these 3 age phases are represented as step functions it would be expected that in a biological system any changes would be continuous rather than discrete. This use of step changes, whilst being an approximation to reality does, however, enable us to make predictions readily and gives an impression of changes over time which helps to demonstrate developmental patterns. These age phases were identified as patterns in the non-parametric analysis by the troughs which occurred in the number of follicles at intermediate stages II-IV. Thus there does not necessarily have to be any physiological significance to these phases

The first phase of post-natal follicular development as identified by the model spans from birth to 20 days of life and it corresponds to the transition identified in the study of Faddy *et al.*, (1983). During this phase the numbers of follicles in the non-growing pool (stage I) declined rapidly as a result of death and recruitment into the growing population. The majority of these losses were due to dying follicles, and these have been identified histologically by other workers (Byskov and Rasmussen, 1973). It was difficult to accurately estimate the death of small follicles by morphological methods and it is in this area that the mathematical model can be particularlly useful. More than twice as many small follicles die than enter development during this early phase and if this rate were to continue throughout life, sterility would be

rapidly reached. The cause of these deaths can not be identified by this study but this phenomenon of massive death resembles programmed cell deaths occurring in other organs.

Since the death rate of these follicles is reduced at later stages it could be hypothesised that follicle density and circulating hormone levels play an important role in these deaths. The pituitary gonadotrophins circulate in high concentrations during juvenile life with the circulating levels of FSH and LH, reaching peak levels during days 9-15 of postnatal life (Stiff *et al.*, 1974; Dullaart *et al.*, 1975). Thus it is unlikely that it is a gonadotrophin deficiency that is responsible for these substantial early deaths. Indeed it has been argued (Hage *et al.*, 1978) that the high levels of FSH present in rodents before 20 days of age may be responsible for the greater number of follicles starting to grow and for an increase in the rate of follicle growth at these early ages (Pedersen, 1970a). Studies on follicular dynamics in the mutant strain of hypogonadal mice (Halpin *et al.*, 1986) have shown that low levels of gonadotrophins do not result in increased death from the primordial pool at early ages.

During this phase, follicles begin to grow but it is still unclear what initiates growth of primordial follicles. It is thought that this process is independent of the influences of the pituitary gonadotrophins although fewer primordial follicles begin their growth in calorie restricted or hypophysectomized animals (Nelson *et al.*, 1985; Jones & Krohn, 1961b). Evidence that primordial follicles in immature ovaries require a "priming effect" by gonadotrophins to initiate growth has been obtained from studies on mice injected with antisera to

gonadotrophins on the day of birth (Eshkol *et al.*, 1970) and on anencephalic human fetuses, in which the hypothalamus and the posterior lobe of the pituitary are absent or their anatomical relationship is grossly abnormal (Baker & Scrimgeour, 1980). Such studies indicate that initial follicle growth is inhibited in the absence of the pituitary gonadotrophins. The precise role of gonadotrophins in the regulation of growth and death in the immature ovary is still unclear but it is known that follicles are capable of initiating and maintaining growth as far as stage IV independently of the pituitary gonadotrophins (Jones & Krohn, 1961b; Faddy *et al.*, 1983) although there is some evidence of pre-antral follicle development in rodents being modulated by gonadotrophins (Arendsen de Wolff-Exalto, 1982; Halpin *et al.*, 1986).

Although the death rates in the pool of small non-growing follicles were high in the first phase they were much reduced in the following two phases. In contrast to this decline in death rates the rates at which follicles begin to grow (migration from I-II) did not show any significant changes during the ages studied and this is consistent with the view that the initiation of growth is independent of changes in gonadotrophins and also of the presence of other follicle types and corpora lutea. The sum of the rates of primordial follicle death and growth determines the net rate of loss from the follicle store. Other workers have shown that the rate of loss from this store is not fixed and is slowed in hypophysectomized animals and in intact animals on restricted diets (Jones & Krohn, 1961b; Nelson *et al.*, 1985).

The influence of ovarian steroids could also be important in early follicular development as has been suggested in the early period of

oogenesis (Arrau *et al.*, 1983), although high affinity binding to alpha-fetoprotein would minimise effects during the first few weeks of life (Linkie & La Barbera, 1979). Increasing attention is being focussed on the role of paracrine and autocrine factors in the regulation of ovarian function and studies on the interaction of gonadotrophins with these factors may shed light on mechanisms influencing the dynamics of follicle growth (Tonetta and diZerega, 1986).

The model predicts that there will be substantial deaths at stage II of follicular development but these cannot be substantiated by histological observations since atresia at this stage of development is difficult to detect morphologically because of the speed of the degenerative processes. The model does not predict any deaths at the intermediate stages of follicle growth. Follicular atresia has been rarely detected at the intermediate stages of follicular development (Oakberg, 1979) although a failure of detection may be indicative of poor criteria to define atresia of small follicles morphologically. It is known that the levels of gonadotrophins and sex steroids are involved in the atretic processes. Hypophysectomy results in atresia of antral follicles (Ingram, 1953; Jones & Krohn, 1961b) and the administration of oestrogen or gonadotrophins to hypophysectomized animals reduces the rate of atresia (Jones & Krohn, 1961b; Goldenberg *et al.*, 1972; Harman *et al.*, 1975). Other studies have shown that androgens increase atresia, (Hillier & Ross, 1979; Nandedkar, 1981), whereas PMSG and FSH prevented it (Peters *et al.*, 1975; Peluso & Steger, 1978; Hay & Moor 1978; Byskov, 1979b; Braw & Tsafriri, 1980b).

The rates of migration through successive stages (II-V+) are high in the first phase with stage V+ follicles emerging by day 6 and being present in all animals by day 9. During phase 1 some follicles develop to antral stages but since there are no ovulations these follicles terminate in atresia. The percentage of stage V+ follicles showing signs of atresia is not significantly greater than that observed in phases 2 and 3 however, the percentage of stage VIa follicles that are atretic is significantly greater.

Studies using the labelled mitosis technique (Pedersen, 1969) have shown that follicles grow faster at early stages of life , with the development of unilaminar follicles to antrum formation taking 10 days in 7 day old mice compared with 16 days to reach the same stage in 28 day old mice (Pedersen, 1970b). This rapid growth of the larger stages at earlier ages may reflect the elevated prepubertal levels of gonadotrophins, FSH and LH. Although antral follicles are present at an early age in the ovary, the immature mouse ovary is not responsive to the ovulation inducing effects of exogenous gonadotrophins until day 20 (Gosden, 1985a). Further changes in follicular dynamics may be occurring at this early age but the model is unable to detect these. The rates of movement and death observed at these ages could not be sustained throughout life as sterility would be rapidly reached. The ovary is sensitive to many changes at these early ages and day 20 marks the beginning of a phase when influences from large growing and degenerating follicles might be expected.

The second phase of post-natal ovarian development extends from 20 to 60 days of age. This age span encompasses the time at which puberty

occurs (approximately 30 days of age) in CBA/Ca mice. This phase is a transitional one between the time when the ovary is refractory to ovulation inducing stimuli on the one hand and the development of regular oestrous cycles on the other. The numbers of stage II follicles reach an early peak in this phase and are reflected by corresponding peaks in the stages III and IV as a result of continuing recruitment. Stage V+ follicles attain numbers during this phase which are maintained approximately constant until at least 100 days old. The percentage of VIb follicles that are atretic is significantly lower during this phase than in the previous phase.

The third phase identified by the model covers the ages when optimal fecundity and most regular oestrous cycles are attained (Kennedy & Kennedy, 1972; Nelson *et al.*, 1982). During these ages there is steady recruitment to maintain follicular constancy at stage V+. There is a large excess of growing follicles at young ages compared with the requirements for a normal ovulatory quota of oocytes and so this would suggest that the process of recruitment into the large preovulatory sub-population must be finely controlled. The fairly constant rate of initiation of growth from I-II suggested by the model indicates that this is not the stage primarily responsible for control. It would seem from this study that control is being exerted at stages II-III and III-IV, these recruitment rates being reduced in phase 3 to about 15% of those in phase 1. The mechanism whereby recruitment of smaller follicles is modulated is not clear and as yet a physiological basis for the suppression of recruitment of preantral follicles acting either directly or indirectly via the pituitary gland has not been established,

although there is evidence of factors suppressing the recruitment of larger stages.

The mathematical model was not designed to provide information about the dynamics of the sub-population of follicles which make up the stage V+, i.e. stages V, VIa and VIb. as their numbers are comparatively low. The information obtained by scoring for atresia in these groups of follicles indicate that at pro-oestrus the incidence of atresia is greater at early antral stages than at large antral stages. The pattern of atresia found in these cycling animals is similar to that found by other workers (Numazawa & Kawashima, 1982). The mean number of VIb follicles found in mature animals is 9.5 and with 15% atresia this would leave a mean of 8 follicles available to undergo final maturational changes in preparation for ovulation.

This study involved a longitudinal analysis of follicle utilisation using a mathematical modelling approach. The problem when dealing with a continuous dynamic system is to transform data obtained from static points to a continuous system which can be described precisely by mathematical parameters. Pulse-labelling with tritiated thymidine with subsequent examination by autoradiography is an alternative method which allows the continuous process to be examined. This can only be carried out within a short time course and has many interpretational problems. If we wish to extend our knowledge of follicular dynamics in other species where histological material was available then the mathematical approach would be the only practical technique as differential follicle counts could be obtained. Furthermore it has been indicated by other studies that follicle death plays an important role

in the regulation of the follicular population as a whole and this can only be reliably estimated by the mathematical approach. The morphological identification of dying follicles at stage I is particularly difficult.

The results obtained from the application of mathematical modelling to follicular dynamics should not be considered of value as simply a means of generating numerical values. This is not the purpose of such a technique, rather it must be stressed that it is a method which detects patterns. Through the emergence of certain patterns in follicular dynamics it is possible to infer how the changes in follicular dynamics occur by reference to existing knowledge of the changing hormonal environment and with other variables such as the presence of large follicles and corpora lutea. Such studies help in identifying levels at which control mechanisms may be operating.

Further studies should be carried out to investigate some of the patterns that have emerged from this study. The variation observed in numbers of follicles present at birth is interesting and it is not known whether there is a relationship between this number and the number of oestrous cycles throughout life, the manipulation of the follicle pool and longitudinal study of smear patterns would be required to answer this. The age changes observed in follicle growth and death could be studied by transplanting ovaries from immature and adult mice reciprocally and counting the number of follicles. Such studies are time consuming and laborious and perhaps other methods will be required to study the biological variables responsible for the observed patterns of follicle growth and death.

Chapter Three:

Follicular dynamics in unilaterally ovariectomised CBA/Ca mice.

Introduction.

The removal of one ovary results in a compensation by the remaining ovary to produce the characteristic ovulatory quota of oocytes. This phenomenon has been recognised since the experiments of John Hunter in 1787 showed that a unilaterally ovariectomised pig produced litters of similar size to the control (although fewer litters). In more recent times the effects of unilateral ovariectomy have been studied in several other polytocous species; rat (Arai, 1920; Mandl & Zuckerman, 1951; Peppler & Greenwald, 1970a,b; Peppler, 1971), mouse; (Jones & Krohn, 1960; Biggers *et al.*, 1962; McLaren, 1966; Baker, 1980), hamster; (Chatterjee & Greenwald, 1971; Chiras & Greenwald, 1978), guinea pig (Hemreck & Greenwald, 1964), pig, (Brinkley & Young, 1969), cow, (Saiduddin *et al.*, 1970), sheep, (Land, 1973; Fry *et al.*, 1987).

The phenomenon of compensatory ovulation is cited as evidence that ovarian function is controlled by extrinsic factors and that the species ovulation number is closely regulated (Lipschutz, 1927; 1928). Some workers have looked at the effect of unilateral ovariectomy on somatic development (Arai, 1920), whilst others have been more concerned with the effect of surgery at specific stages of the oestrous cycle (Bast & Greenwald, 1977) and with the mechanisms involved in the compensatory response (Fry *et al.*, 1987). Evidence exists for two possible mechanisms involved in compensatory ovulation:

1) an increased rate of recruitment of the developing follicles

2) a decreased rate of atresia among larger stages

The mechanisms have been presumed to depend on the interactive relationship between the hypothalamus, anterior pituitary gland and the

ovary, involving trophic hormones and negative feedback pathways. Two hypotheses have been suggested by Bast & Greenwald (1977) to account for these.

1) The amount of pituitary gonadotrophin FSH & LH, available for utilisation by developing follicles is doubled by the removal of an ovary. The changes, if any, in gonadotrophin levels are not important.

2) An increase in FSH secretion as a result of the reduction in negative feedback by oestrogen and inhibin. This rise in FSH may permit recruitment of follicles that might otherwise have undergone atresia (Hirshfield & Midgley, 1978).

These mechanisms assume that the level of control leading to compensatory ovulation exists at the large stages of follicle development; this is reasonable given the short latency of this response. It is, however, unclear whether preceding follicle stages are influenced, particularly the small follicles in the primordial population which provide a reserve. An increased utilisation of small follicles would have implications for the timing of sterility.

This present study was designed to examine the dynamics of ovarian follicles from primordial to large antral stages by utilising the technique of compartmental analysis. By comparing the findings with the model obtained from normal animals (see chapter 2) it was hoped that any effects of unilateral ovariectomy on the growth and death of follicles could be identified. Additional quantitative morphological studies were carried out to estimate the rate of atresia of larger

follicles, which cannot be precisely estimated using the present model because of the low number of follicles at these stages.

Materials and Methods:

Animals:

The animals used in this study were inbred CBA/Ca mice obtained from our own breeding colony and reared in the Faculty Animal Area of the University of Edinburgh. The animals were selected from litters within a size range of 5-10 and weaned at 21 days. After weaning animals were housed in pairs and provided with a pelleted diet and water ad libitum under a photo-period of 14 hours beginning at 0700h GMT in a thermostatically controlled environment at 21°C. Vaginal smears were taken daily from animals aged over 39 days and those in which pro-oestrus occurred at 41-43 days were selected for unilateral ovariectomy or sham operation. A total of 33 animals were unilaterally ovariectomised and 5 animals were sham-operated. On the basis of finding similar follicle counts in the sham operated group and the much larger series of "control" animals from the work described in chapter 2, the two sets of data were combined. The control and unilateral ovariectomised group were reared concurrently under identical conditions in the same animal house.

Surgery:

The animals were anaesthetised with Avertin (tribromoethanol, Winthrop Laboratories) at a dose of 0.005g per 10g body weight administered intra peritoneally. A dorso-lateral incision was made in the skin and the body wall. Using a dissecting microscope and the "cold" light of a

fibre optic system the ovarian fat pad was located and exteriorized to reveal the ovary. The ovarian blood vessels and oviduct were ligated with fine suture close to the hilum and the ovary was removed. The body wall and outer skin were sutured and animals were returned to the holding room. Either the right or the left ovary was removed in alternate animals.

Collection of material:

Animals were sacrificed by cervical dislocation on the day of pro-oestrus at various times after operation, ranging from 4 to 50 days when they were 48-95 days old. Smears were taken prior to both the day of operation and the day of sacrifice to determine the day of pro-oestrus. The ovaries were removed and fixed in aqueous Bouin's fluid, dehydrated in graded alcohols and embedded in paraffin wax. Each block was serially sectioned at 7μm and stained with haematoxylin and eosin.

Follicle Counts:

Follicles were classified according to the number of granulosa cell layers surrounding the oocyte, following the scheme of Mandl & Zuckerman (1951) (see chapter 2, page 25). All counts were done 'blindly' using coded slides. Differential follicle counts of follicle stages I (primordial), II (One layer of cuboidal granulosa cells), III (two layers of granulosa cells) and IV (three layers of granulosa cells) were obtained by counting those follicle when a nucleolus appeared in every tenth section. The estimated total numbers of follicles were obtained by multiplying the raw counts by the sampling frequency and a correction factor to compensate for overcounting (Abercrombie, 1946).

Follicles in which four or more layers of granulosa cells were present were classified as stage V+ for the purpose of modelling. They were also divided into three sub-groups: stage V where more than four layers of granulosa cells were present but fluid-filled spaces absent, stage VIa follicles in which fluid-filled spaces have appeared but an antrum has not yet formed and VIb large follicles in which the spaces have coalesced to form the continuous antrum and the oocyte is surrounded by the cumulus mass. Every section was examined to estimate the numbers of these stages.

Atresia:

Follicle stages V, VIa and VIb were closely examined for signs of degeneration ("atresia"). The criteria for atresia were based on the morphological appearance of the granulosa cells and of the oocyte, two categories of atretic follicles were defined.

Type 1: 3-20 pyknotic granulosa cells observed in the largest cross section.

Type 2: Greater than 20 pyknotic cells in the largest cross section and/or obvious degeneration of the oocyte or its membranes.

Data analysis:

Differential follicle counts obtained from animals over an age span of 47-95 days were analysed by compartmental modelling (Faddy *et al.*,1976; Faddy *et al.*, 1983; Halpin *et al.*, 1986). The assumptions and formulation of the compartmental model as applied to follicular dynamics is dealt with in the previous chapter (pages, 28-31). As with the longitudinal study of normal intact animals, the data obtained from the unilaterally-ovariectomised group were analysed by non-parametric

regression techniques to aid in the identifying patterns which would then guide the fitting of the parametric model.

The proportions of atretic follicles at stages V, VIa & VIb from the unilaterally ovariectomised were compared with those in the normal intact group using an non-parametric analysis of variance by ranks test, Kruskal-Wallis. This test does not assume a normally distributed population and so is preferable in this situation to the standard parametric tests (Siegel, 1956).

Results:

The data obtained from differential follicle counts are presented in figure 3.1 a-e. Each point represents one animal and the raw counts are presented in the figure together with the non-parametric and parametric regression curves to describe follicle dynamics between 42 and 100 days of age. It can be seen from these data that similar variation exists compared with that in intact animals (Fig. 2.4a-e. chapter 2, page, 33-34). The parameters obtained from the longitudinal study of normal intact animals were applied to the new data, but the fit was unacceptable which suggested that the migration and/or death rates had been altered in the experimental group. Consequently, new parameters had to be formulated to describe the behaviour of follicles after unilateral-ovariectomy (figure 3.2).

The data have been divided into two phases to improve the precision of the fitting to the model. The first phase extended from 42-67 days and the second from 67-100 days. The data were apportioned to these phases because of the behaviour of the stage II follicles which showed a bi-phasic form.

Figure 3.1a-e:

Graphs a-e (pages 60 & 61) represent the number of follicles (stages I-V^{+}) in 33 CBA/Ca mice after unilateral-ovariectomy. Animals were operated on between 41-43 days post partum on the day of pro-oestrous. The data were obtained by differential counting of follicle stages. The dotted line corresponds to the fit of the parametric curves obtained from the application compartmental modelling to the data. The dashed line corresponds to the non-parametric regressions.

Each graph does not always have 33 points this is because the same value has occurred more than once.

Fig. 3.1a

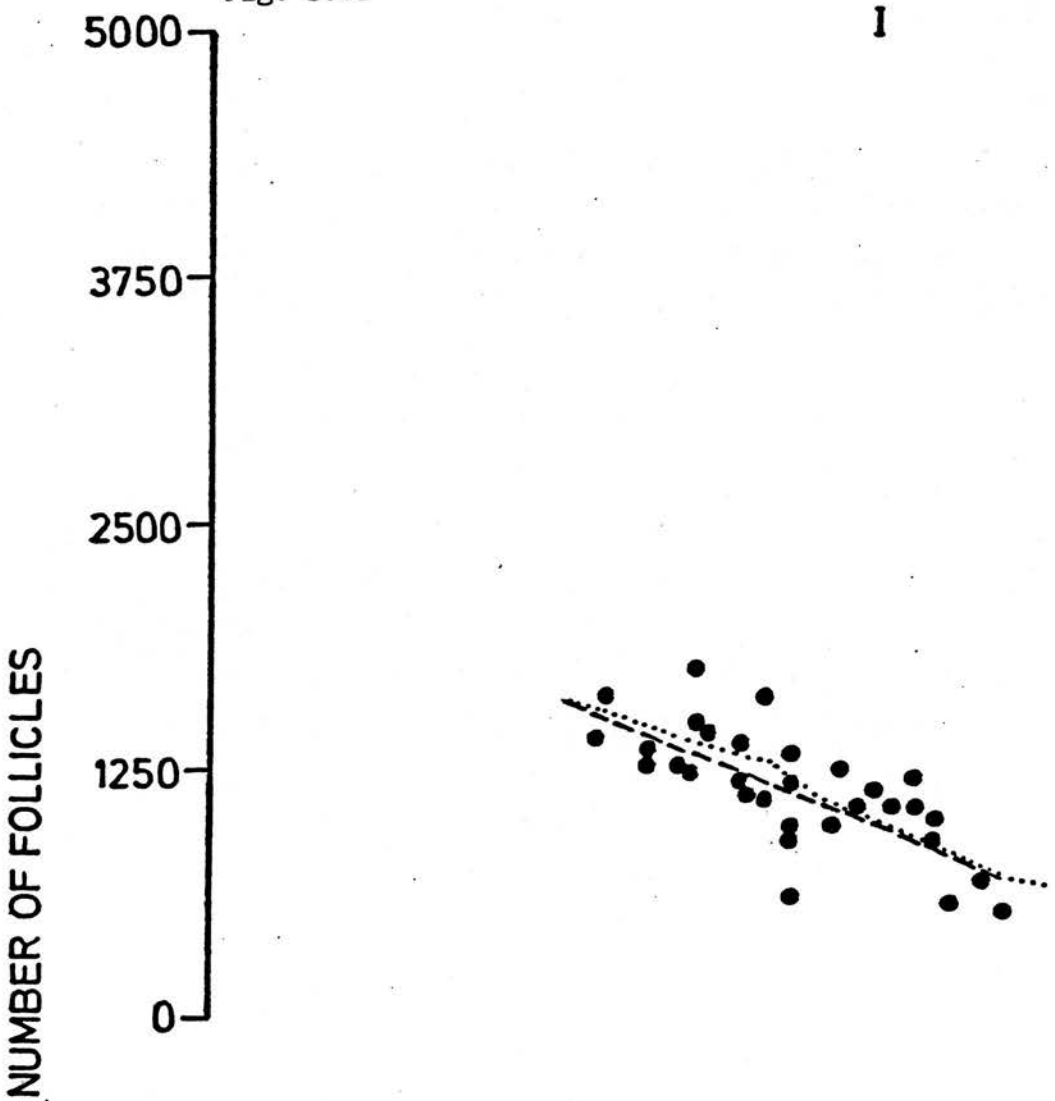


Fig. 3.1b

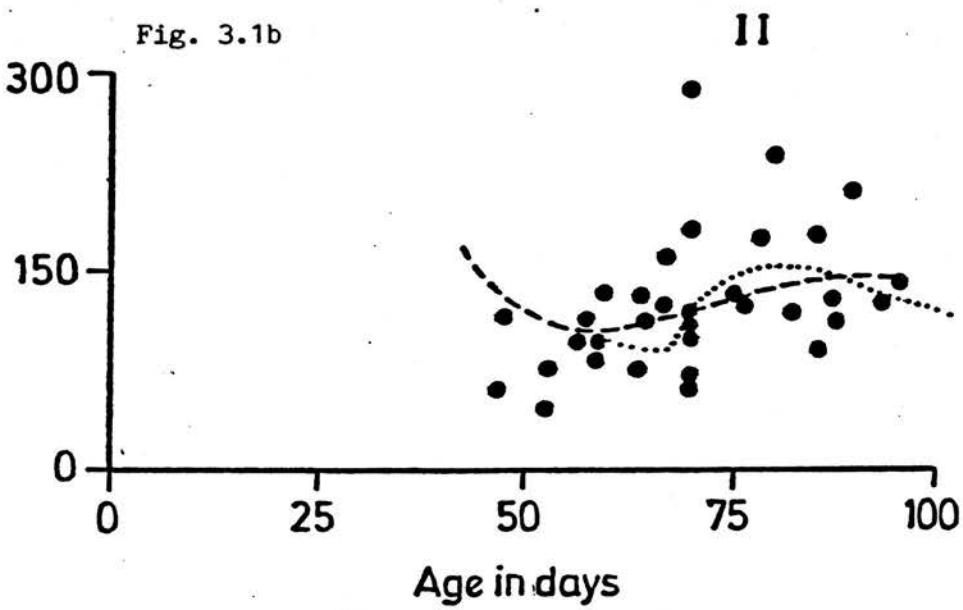


Fig. 3.1c

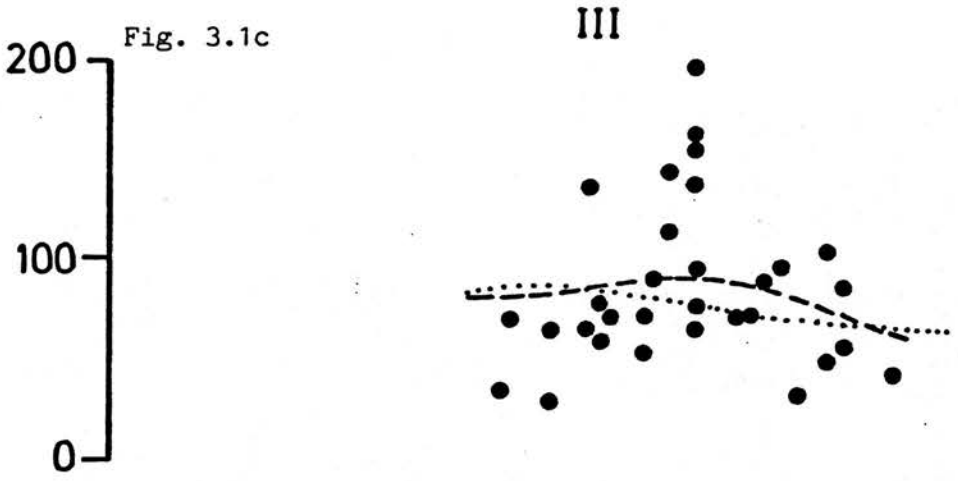


Fig. 3.1d

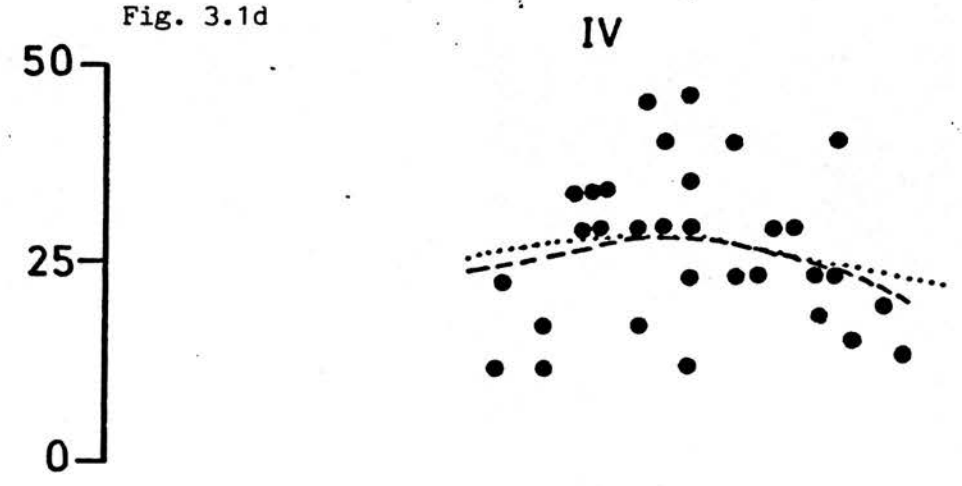
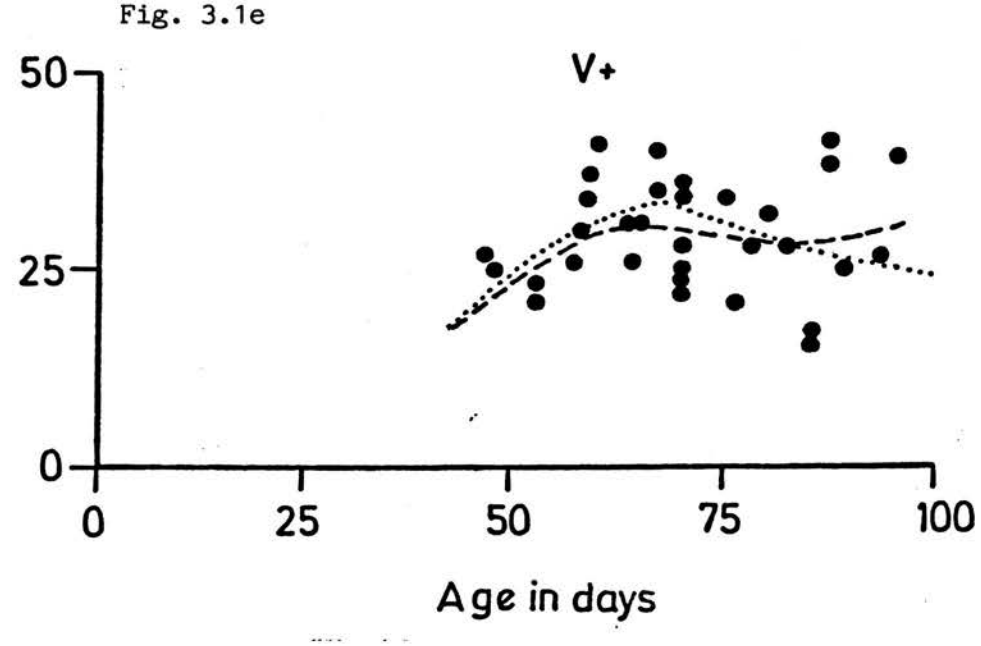
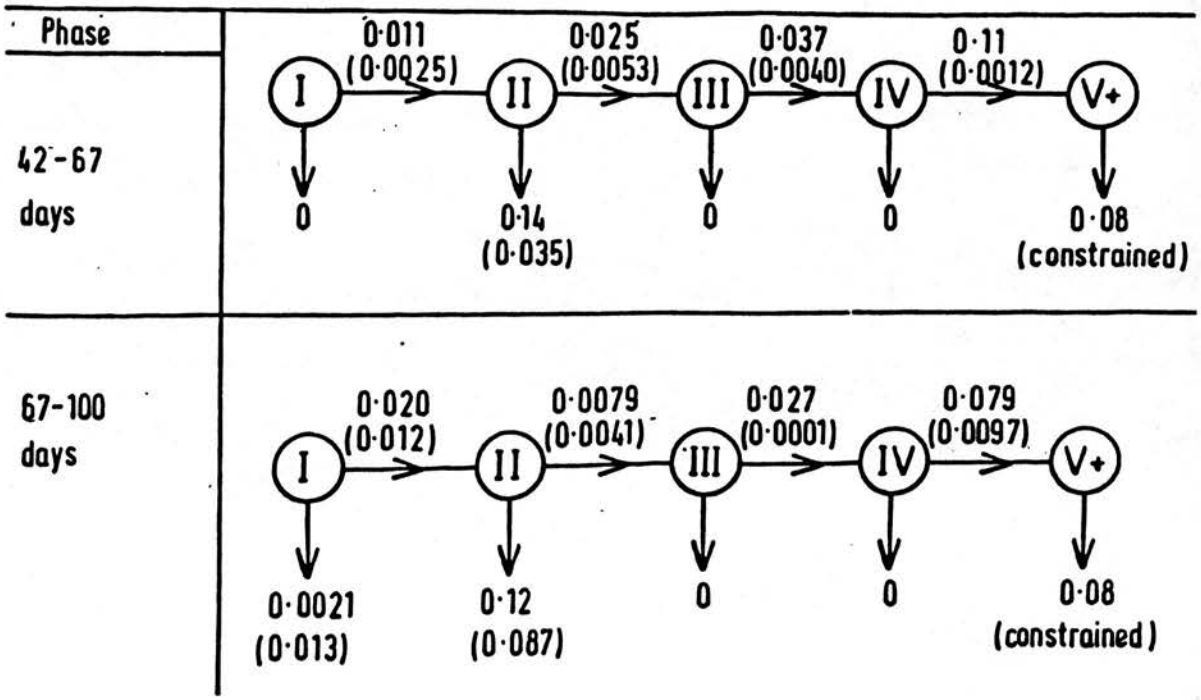


Fig. 3.1e



NUMBER OF FOLLICLES

Figure 3.2:
Parameter estimates obtained for growth (migration) and death (atresia) through five stages of follicular development in unilaterally ovariectomised animals. Two age phases are indicated. Migration from stage V+ has been constrained to correspond with mean ovulation number.



Phase 1: 42-67 days

There is a steep age-related decline in stage I follicles compared with control ovaries. The rate at which follicles leave the primordial pool by both death and growth to the following stages during the post-operative phase is similar to that during phase 2 of the normal animals. The rate at which follicles enter from the primordial pool of follicles to the growing phase (migration from stage I-II) is significantly greater than that estimated for intact animals at the same age, this being .011 in the unilaterally ovariectomised group compared with .0087 in the intact group. This rate represents about 17 follicles starting to grow each day in the unilateral ovariectomised compared to 28 in the intact animals where twice the number of reserve follicles (stage I) are present. Surprisingly, follicle deaths in primordial stages were not detected at this phase. This does not necessarily mean that death rate was zero because a low rate might go undetected.

There was a marked decline in the numbers of stage II follicles. The implication is that more follicles were leaving this stage than entering it. At 50 days of age, a mean of 17 follicles entered the growing stages whereas a mean of 21 left stage II per day. Death from stage II follicles was detected and of those follicles leaving stage II 84% died leaving only 3 follicles surviving to reach the next stage. This result resembles the situation in the immature intact animals (during phase 1) in which 76% of stage II follicles were dying (chapter 2). The death rate at stages III & IV was negligible, consequently 3 follicles leaving stage III per day reached stage IV and eventually produced 2-3 at stage V+. Since slightly more follicles entered both

stages IV and V than left them, there was a corresponding rise in the numbers of those larger stages. The accumulation of stage V+ follicles was also due to the greater number of follicles entering this stage daily than leaving it. (see table 3.1)

Phase 2 (67-100 days):

During this period the rate of loss of stage I follicles was increased compared with both the earlier post-operative period and intact animals. This increase was due to both an increase in growth rate to next stage and deaths from stage I. A high death rate from stage II was indicated and Stage I and II should be considered together in this case since all these values have high standard errors, but the model is detecting deaths at small follicle stages.

These parameters estimate that at age 70 in the operated group, of the 26 follicles leaving stage I 23 will proceed to the next stage but of 15 follicles leaving stage II only 1 will proceed to the next stage. This surprising result was predicted by the optimal fit of the model having attempted alternative balances between growth and death rates.

Few follicle deaths occur at the intermediate stages III-V+ at ages 67 - 100 days. There is an increased rate of movement through the stages, this being especially marked between stage IV-V+, where the rate is .079 compared with .042 in the intact animals. This doubling of the recruitment rate into the pre-ovulatory cohort anticipates the doubling of the ovulation rate. When actual numbers are estimated we find that an average of 2 follicles leave stage III per day and reach stage IV.

Table3.1:

Average numbers of follicles leaving the stages (compartments) per day, either by growth to the next stage or death after unilateral ovariectomy. The dividing line represents the two age phases indicated by the model.

Age	I→	→II	II→	→III	III→	→IV	IV→	→V→	V→
49	16	16	47	3,0	3,0	3,0	2,7	2,7	1,9
56	15	15	39	2,5	2,9	2,9	2,9	2,9	2,3
63	14	14	35	2,2	2,8	2,8	2,9	2,9	2,5
70	26	23	15	0,9	1,9	1,9	2,1	2,1	2,5
77	22	20	19	1,1	1,8	1,8	2,0	2,0	2,3
84	19	17	18	1,1	1,7	1,7	1,9	1,9	2,1
91	16	14	17	1,0	1,6	1,6	1,8	1,8	2,0
98	14	12	15	0,9	1,5	1,5	1,7	1,7	1,9

Of the mean of 0.9 follicles entering stage III a mean of 1.9 are leaving by growth to stage IV, this results in a slight decline at stage III. Stage IV remains steady as the numbers leaving are balanced by the numbers entering this stage and this being a mean of 2 at age 70 days.

Figure 3.3 gives the total number of healthy and atretic stage V, VIa and VIb follicles at ages 42-66 and 67-100 days in unilaterally ovariectomised and intact animals. At ages 67-100 days the mean total number of follicles at stages V and VIa in normal animals are significantly higher than in unilaterally ovariectomised (uni-ovx) animals but the number of atretic follicles are less in the uni-ovx group than in the normal group. This results in a mean of 8 non atretic stage V follicles in normal animals compared with a mean of 7.6 in unilateral ovariectomised animals and a mean of 13 non atretic stage VIb follicles in normal animals compared with a mean of 10.2 in uni-ovx animals. The numbers of stage VIb follicles are not significantly different between normals and unilaterally ovariectomised animals these being 9 and 8 respectively with a mean of 1.6 and 1.4 atretic follicles.

Figure 3.4 presents the same data as in the previous figure but in terms of percentage of atresia at stages V, VIa and VIb at ages 42-66 days and ages 67-100 days in normal and uni-ovx animals. The percentage of atretic follicles at stage V has been reduced by about 9% in the uni-ovx group at ages 42-66 days compared with the normal animals. There is no significant difference in percentage atresia at stage VIa between normal and uni-ovx animals aged 42-66 days.

Figure 3.3:

Mean (+ S.E.M.) number of healthy and atretic follicles at stage V, VIa and VIb in normal and unilaterally ovariectomised animals aged 42- 66 days (top) and aged 67- 100 days (bottom).

Split in ages is equal to the two age phases identified by the model for follicular dynamics in unilaterally ovariectomised animals. Normal intact group corresponds to animals used in the study described in chapter 2.

controls 42-66 days n=8
controls 67-100 days n=18

Uni-ovx 42-66 days n=14
Uni-ovx 67-100 days n=19

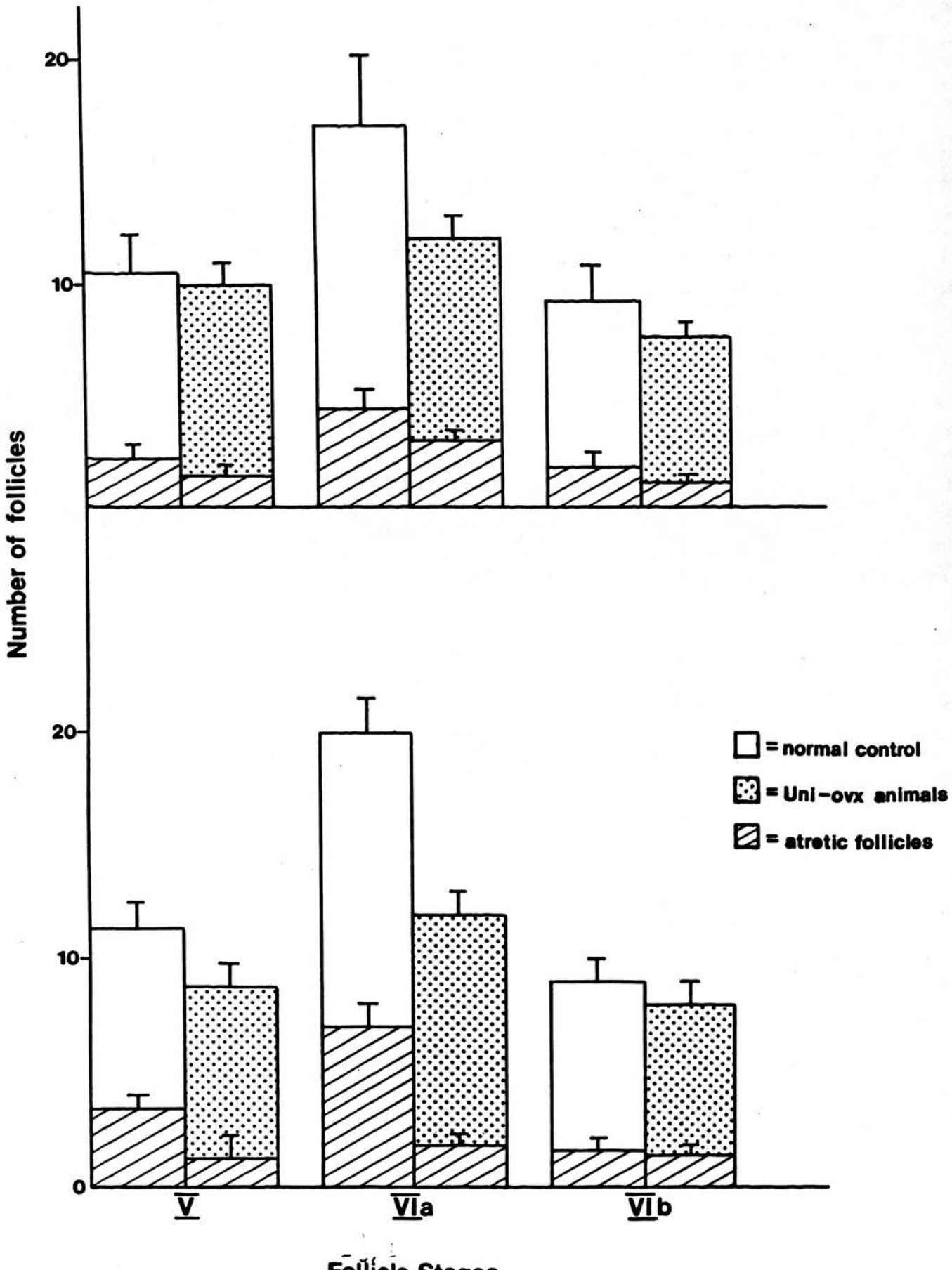
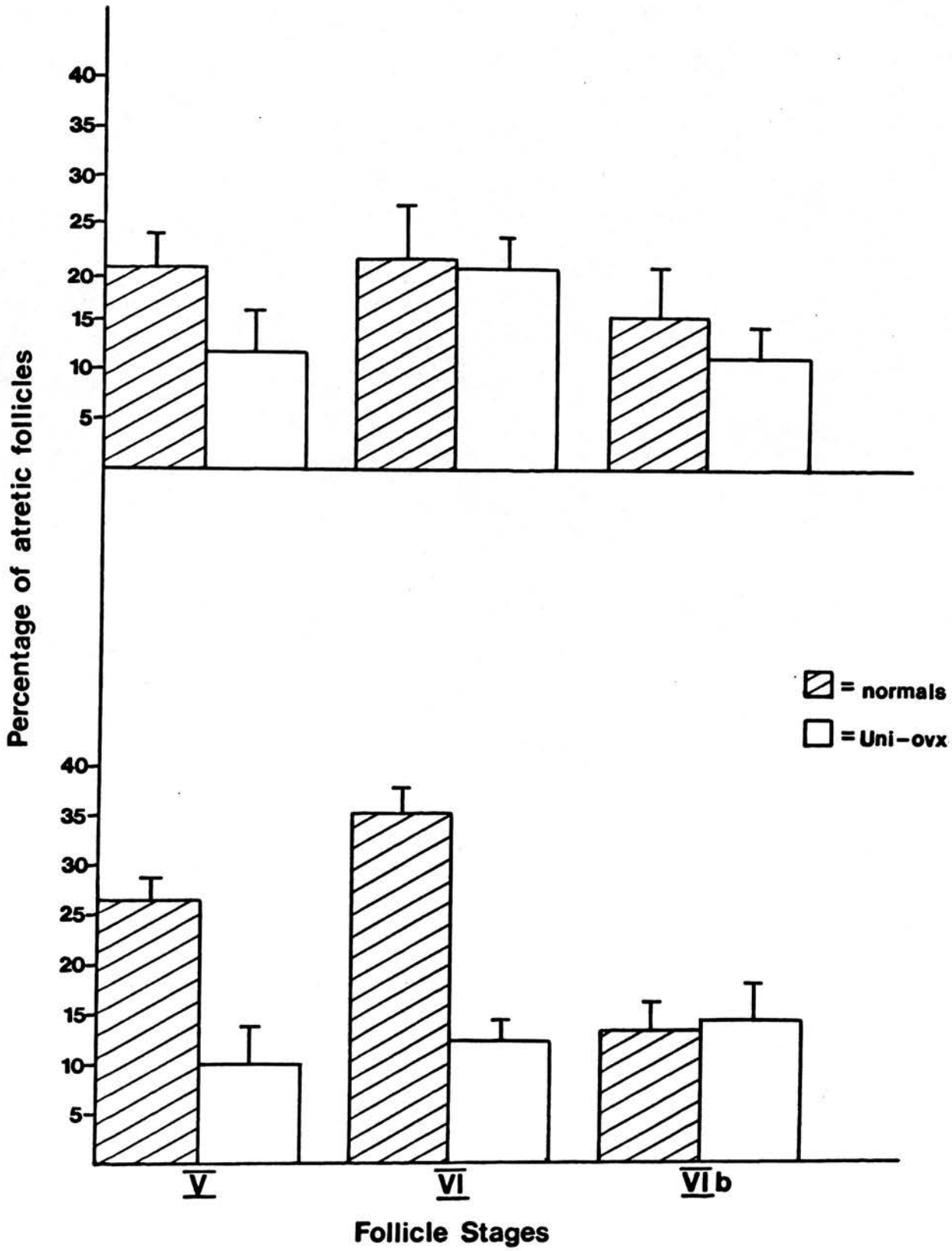

20
10
20
10
0
Number of follicles
= normal control
= Uni-ovx animals
= atretic follicles
V
VIa
VIb
Follicle Stages

Figure 3.4:

Mean (+S.E.M.) percentage of atretic follicles at stage V, VIa & VIb in normal intact and unilaterally ovariectomised animals aged 42-66 days (top) and 67-100 days (bottom).



A decrease of around 4% is found at stage VIb in unilateral ovariectomised animals.

In comparison with ages 42-66 days those animals at 67-100 days show a marked reduction in the percentage of atresia at stages V and VIa but no significant difference at stage VIb when compared to normal animals at a similar age group.

Discussion:

Since the pioneering work of J. Hunter (1787) it has been known that unilateral ovariectomy leads to compensatory ovulation in the remaining ovary. The superovulatory response presents fundamental questions of how the process of follicular recruitment has been altered to match the new demands of ovulation and the long term consequences for ovarian function. The increasing numbers of large follicles and corpora lutea leads to compensatory hypertrophy (Arai, 1920; Mandl & Zuckerman, 1951; Jones & Krohn, 1960). These changes are well known but the mechanisms responsible for compensatary ovulation require clarification. There may be differences in the mechanisms responsible for compensatory ovulation depending on the time elapsed since operation, between species, and even between spontaneous and induced ovulators (Fleming *et al.*, 1984). The present study has examined the follicular dynamics of adult mice after removing one ovary with the particular aim of identifying stages involved in the recruitment of extra follicles.

Two major concepts exist to explain the control of events after unilateral-ovariectomy. Firstly, a fall in the circulating levels of ovarian steroids result in an increased output of gonadotrophins which

leads to increase in follicle growth. Secondly, the level of gonadotrophins may be more or less unchanged but the effects are increased because only half the amount of target tissue remains. The increased ovulation rate of the single ovary remaining after surgery could result from at least three different mechanisms:

1) A decrease in the number of antral follicles lost by atresia

2) An increase in the rate of loss of primordial follicles

3) An increase in the growth rate of preantral follicles.

There is some evidence in rats that additional ovulations occurring after unilateral-ovariectomy occur from follicles that would otherwise have become atretic at met-oestrus and that increased levels of FSH at the preceding oestrus provide a stimulus to their development (Welschen *et al.*, 1978; Otani & Sasamoto, 1982; Hirshfield, 1982, 1983). A decrease in atresia has also been observed in rabbits and hamsters following unilateral-ovariectomy (Desaive, 1949; Greenwald, 1961). This hypothesis does not predict and may not require changes within the preantral population of follicles and such a mechanism would have little effect on long term fecundity. An increased rate of depletion from the primordial pool of follicles has been observed during the first four weeks after unilateral-ovariectomy in mice (Baker *et al.*, 1980). If this effect continued and was an important mechanism for compensatory ovulation there would be far reaching implications for fecundity.

Several studies on unilaterally-ovariectomised animals have found an increase in the number of pre-antral follicles reaching preovulatory size, e.g. in guinea pigs (Hemreck & Greenwald, 1964), ewes (Mallampati & Casida, 1970; Dufour *et al.*, 1979), rats (Peppler & Greenwald, 1970b;

de Reviers, 1987) and mice (Baker *et al.*, 1980). A study of gerbils after unilateral ovariectomy did not indicate any change in preantral follicle numbers, (Norris & Adams, 1982) but this does not necessarily indicate that the dynamics are constant. Differences in findings could be as a result of species differences and of differences in the length of post-operative period studied. If a longer period was studied changes in the preantral population of follicles are more likely to be detected since their growth is slower. In a study of the unilaterally ovariectomised sheep changes in the preantral population of follicles were not evident until 70 days following treatment (Dufour *et al.*, 1979). Clearly the time course of events will vary between species.

The results from this study indicated significant differences in follicular dynamics in animals with one ovary compared with those with two since data obtained from this study could not be fitted to the model derived for normal animals. The results indicated changes at each of the suggested levels of control but the effect of these changes may not be constant as indicated by age dependency in the model. The model divided the results into two phases ranging in age from 42-67 days and 67-100 days. The rate of movement out of stage I during phase 1 is not significantly different from that of intact animals except that in the operated group all follicles proceeded to the next stage of development as a zero death rate is detected this being indicative that deaths from this stage are insignificant post-operatively. Substantial deaths were, however, detected in stage II follicles. It is difficult to be specific about these deaths since standard errors were large for these parameters. Histological evidence of these deaths can not be provided as only 'healthy' follicles at these

stages were counted and no objective criteria exist for the identification of small follicle atresia and this problem was pointed out in the past and continues to exist (Zuckerman, 1951; Jones & Krohn, 1960). The model does not predict deaths at intermediate stages (III & IV) but the most marked result is that the model predicts an increased rate of movement of follicles from stage IV-V+. Similar numbers of follicles entered stage V+ during this phase compared with controls thus providing sufficient numbers for the compensatory response.

Since the latency to compensatory ovulation after unilateral-ovariectomy is only a 2 or 3 days it has been concluded that recruitment of follicles which would have become atretic must have occurred as a short term mechanism. If recruitment from the primordial pool is unchanged in unilaterally ovariectomised animals then additional follicles are presumably derived from follicles which would otherwise have become atretic. In the short term treatment group there is reduced atresia at stage V follicles this being the stage immediately preceding antrum formation, but no significant difference existed between the incidence of atresia of follicles at subsequent stages VIa and VIb. It would seem that the increased rate of growth from stage IV-V+ and a reduction in atresia provides enough follicles for the compensatory response in animals from 4 to 26 days after unilateral ovariectomy.

After 67 days of age (corresponding to around 25 days after operation) the rate of initiation of follicles in the operated group from stage I had increased compared with that of intact animals at a similar age. Evidence for a more rapid loss of oocytes has been indicated in the rat (Mandl & Zuckerman, 1951) during the first few days after unilateral

ovariectomy and in the mouse (Baker *et al.*, 1980). The number of follicles reaching stage II after 67 days is greater than in intact animals and at first sight this would appear to help produce greater numbers of follicles required to bring about the compensatory response. This increased recruitment of stage I follicles in the operated group was negated by deaths at stage II of development which, as in the earlier phase, are exceptionally high. The deaths of small follicles result in a reduction in the numbers of follicles proceeding to stage III in the operated group compared to intact animals. No significant difference in the subsequent rates of movement or death upto stage IV were observed but there was a significant increase in the rate of movement from stage IV to stage V+ in unilaterally ovariectomised animals.

Although growth rate into V+ is increased the actual number of follicles reaching this stage has slightly declined compared with the controls, and is not enough to account for compensatory ovulation. In this study a decrease in the percentage of follicles showing signs of atresia was found at stage V and VIa but not at VIb, in animals aged 67-100 days. Thus although slightly less follicles are reaching stage V+ compared with controls, less become atretic, however, this selection process is complete before follicles reach large preovulatory sizes. In conclusion it would seem that in mice 25-60 days after unilateral ovariectomy, that a combination of effects bring about the compensatory response, viz a reduction of atresia at larger stages and an increased rate of movement into stage V+

The observed decrease in atresia may be as a result of increased FSH levels following unilateral-ovariectomy (Hirshfield, 1982). This transient increase in FSH and its effect on large follicles is comparable with that of superovulation following PMSG treatment in which a reduction in atresia has been observed in mouse and rat ovaries (Peters *et al.*, 1975; Peters, 1976, 1979; Braw & Tsafriri, 1980b). An increase in FSH may also be responsible for the increased growth rate of stage IV follicles as in vitro studies on ovarian follicles have indicated that this stage of follicle is particularly sensitive to FSH (Ryle, 1972).

Small transient increases in plasma FSH concentration have been observed after unilateral ovariectomy in a number of species: ewes (Findlay & Cumming, 1977), rats (Ramirez and Sawyer, 1974; Welschen and Dullaart, 1974; Butcher,1977), hamsters (Bast & Greenwald,1977), gilts (Redmer *et al.*, 1984) and heifers (Johnson *et al.*, 1985). These suggest that the removal of one ovary results in a reduction of a negative feedback from the ovaries with the consequence that FSH levels rise. The increased levels of FSH would be expected to increase the number of follicles developing to large antral stages (Welschen, *et al.*, 1978) and thus lead to a restoration of the species characteristic ovulatory quota. It is not clear whether follicular inhibin or steroids (particularly oestradiol) is the most significant negative feedback agent as it has been demonstrated that both steroid free follicular fluid and oestrogens suppress compensatory ovulation in rats (De Jong and Sharpe, 1976; Welschen *et al.*, 1977).

The longer term effects of the gonadotrophins are not clear and other areas such as the role of ovarian adrenergic nerves have been investigated (Gerendai *et al.,1978*; Curry *et al., 1984*). There has been a recent suggestion that compensatory ovulation after unilateral ovariectomy in the ewe may be independent of feedback between the ovary and the release of gonadotrophins from the pituitary gland (Fry *et al.*, 1987). Fry and his co workers found that in hypophysectomised ewes with one or two ovaries receiving equal doses of exogenous gonadotrophin the ovulation rate per ewe was maintained thus they conclude that the higher concentrations of gonadotrophins after uni-ovx are unlikely to be the main causative factors involved in the subsequent compensatory ovulation and suggest that inter ovarian mechanisms and follicular growth inhibitors which act locally on the ovary play major roles in controlling ovulation as indicated by Cahill *et al.*, 1984.

The manipulation of the ovarian system by the removal of one ovary from a pair presents an interesting system to study inter- and intra-ovarian control mechanisms and can be compared with the ageing effect where there is a reduction in follicle numbers whilst ovulation rate is maintained. The reproductive performance of many species declines with advancing age (Talbert, 1978) and it is thought that in some species this may be related to the reduction in the numbers of follicles and, thus, ovulations (Butcher & Page, 1981). When one ovary is removed the cycles are relatively unaffected and compensatory ovulation continues to an advanced age (Peppler, 1971). Comparing this effect of ageing with that in unilateral-ovariectomised animals is complicated by

observations that there are alterations of the hypothalamic pituitary axis with ageing (Butcher, 1985).

In conclusion, the reduced ovarian tissue in young cycling mice affects the population of follicles at all stages and differences may exist over the short and longer term. There may be further changes in follicular dynamics especially in relation to the rate of loss from the primordial pool of follicles over a longer post-operative period not covered by this study and as indicated by Baker *et al.*, 1980. The value of the present study is to identify stages of follicular development that are affected by this experimental manipulation. Some of the observed changes, such as a reduction in atresia, may be due to a direct influence of gonadotrophins but the effects on preantral follicles may be due to a combination of hormonal and intraovarian factors. Elucidation of the mechanisms whereby follicle growth and death rates are modulated will require new experimental approaches to examine the effects of the gonadotrophins, sex steroids and other growth factors at all stages of follicular development. Further study is necessary to separate causal and incidental factors, particularly in connection with the relationship between numbers of large healthy and large atretic follicles with growth and death rate of small growing follicles.

Chapter Four:

Effects of suppressing ovulation by progesterone on follicle dynamics.

Introduction:

It has been known since the beginning of the century that the corpus luteum is required for the maintenance of pregnancy (Fraenkel, 1910). Later it was known that its major hormonal product is progesterone (Allen & Corner, 1930). Whilst comparative physiology has shown enormous evolutionary diversity in the mechanisms for controlling reproductive functions it appears to be a universal fact that during pregnancy ovulation does not occur and this is connected with the actions of ovarian steroids. This ovulation inhibiting effect of ovarian steroids has been the basis for the development of orally active hormonal compounds for contraceptive purposes (Pincus & Chang, 1953).

Measurements of peripheral hormone levels have shown that pulses of FSH and LH are substantially suppressed during human pregnancy (Baird, *et al.*, 1984; Jeffcoate, 1986) but this is much more variable in rodents (Linkie & Niswender, 1972; Murr, *et al.*, 1974a). Whilst it is clear that the ovarian steroids, oestrogen and progesterone, can suppress the maturation and ovulation of large follicles, (Richards, 1980) little is known of their effects on smaller stages of follicles when ovulation is inhibited. It has been assumed that the death ("atresia") of Graafian follicles can explain anovulation but it is not known whether the preantral or the primordial population of follicles are affected.

Follicular growth through pregnancy in the mouse has been studied by Pedersen & Peters, (1971) and they found that new follicles began to grow each day and that follicles of all stages of development, except preovulatory follicles were found. Similar observations of follicle

growth throughout pregnancy have been found in various species: hamster (Greenwald et al., 1967); rat (Schwartz & Talley, 1968); human (Govan, 1970).

It is not clear how the population of follicles are affected by the absence of large ovulatory follicles since relatively little is known about the nature and actions of intra-ovarian factors involved in the regulation of follicular growth. It has been suggested that the size of the population of preovulatory follicles influence the growth rate of smaller follicles perhaps via an inhibitory substance produced by the large follicles (Peters *et al.*, 1973a). Modelling of follicular dynamics in four strains of mice indicated that reduced rate of movement of small follicles observed after puberty was reversed after hypophysectomy (Faddy *et al.*, 1983), thus, changes in the rate of follicular growth were inversely associated with changes in the large follicle population. This information substantiates the suggestion that a factor capable of retarding the recruitment of smaller follicles is released from large follicles.

The aim of this study was to examine the effects on the recruitment of follicles of inhibiting ovulation using progesterone implants. This question contrasts with that of a previous chapter (chapter 3) in which the dynamics of follicles were studied in ovaries which were superovulated as a result of unilateral-ovariectomy. Whilst both of these experimental situations involve changes in extrinsic influences it was hoped that they may reveal whether intraovarian factors are acting to influence follicular dynamics.

Materials and Methods:

Animals:

All animals used in this study were inbred CBA/Ca mice obtained from our own breeding colony. Females were selected from litters ranging in size from 5-10 pups, weaned at 21 days and subsequently housed in pairs in thermostatically controlled rooms at 21°C and under controlled lighting conditions with a photo period of 14 hours beginning at 0700h GMT. A pelleted diet was provided (S.D.S) and water ad libitum. Vaginal smears were taken by lavage from all adult animals for fourteen days prior to surgical procedure to establish that normal oestrous cycles were taking place. These were continued throughout the experiment until animals were sacrificed. Only those animals showing three or more consecutive cycles of 4 days long were selected for experimentation.

Preliminary study:

The purpose of a preliminary study was to establish whether the progesterone implants abolished oestrous cycles and prevented ovulation. Vaginal smears were taken from 24 female CBA/Ca mice aged between 40-45 days for three consecutive cycles. Only those animals showing normal oestrous cycles were selected and distributed randomly between three groups with eight animals per group. The groups are shown below:

Group 1 control group in which animals received an empty silastic implant.

Group 2 Experimental group with one silastic implant containing progesterone.

Group 3 Experimental group with two identical silastic implants containing progesterone.

Implants were made from 1cm lengths of sterile silastic tubing (Dow. Corning, Michigan,U.S.A) with an internal diameter of 1.9mm. Progesterone (4 pregnene-3,20 dione, Sigma, U.S.A.) was packed into the length of the tubing and the ends sealed with a medical adhesive silicone type A (Dow. Corning, Michigan,U.S.A.) preparation for bonding silastic elastomers. Implants were preincubated in a sterile saline solution at 37°C. Animals were anaesthetised using Avertin (tribromoethanol, Winthrop Laboratories) at a dose of 0.005g per 10g body weight and implants were inserted subcutaneously into the nape region. The implants were kept in place for 16 days during which time a record of vaginal smears was collected. At the end of the experimental period blood was taken by cardiac puncture whilst the animal was anaesthetised, after which animals were killed by cervical dislocation. Both ovaries and oviducts were removed and placed in a watchglass of saline. Oviducts were teased apart and examined for the presence of ovulated oocytes using a dissecting microscope at X25. The ovaries were examined for the presence of fresh corpora lutea. Implants were checked at autopsy and on the basis of hormone remaining one implant was effective. The progesterone concentrations in the blood sera were analysed by radioimmunoassay (Fraser, 1983).

Implant Group:

Sixty animals were selected from an age range of 38-42 days post partum. Eight animals were rejected because they displayed abnormal smear patterns, with extended dioestrous type smears. When the

remaining 52 animals reached approximately 55 days of age either a control implant or a single progesterone implant was inserted sub cutaneously. A single implant was chosen because this was found to be efficient during the pilot study. Control implants were inserted into 12 animals and progesterone implants into 40 animals. Vaginal smears were taken from all animals before and after the insertion of the implant. Animals were killed between 5 and 120 days after implant. Since the effective life of these implants for blocking ovulation was around 35-40 days implants were replaced every 30 days. Table 4.1 shows distribution of animals by group, age and days of treatment.

At autopsy ovaries were removed from animals and placed in Bouin's fixative for 24hours, dehydrated through graded alcohols and embedded in paraffin wax. Paraffin wax blocks were serially sectioned at 7μm, mounted and stained with haematoxylin and eosin.

Differential follicle counts:

Every tenth section was examined blindly using coded slides and the nucleolus was used as a marker for counting follicles. Follicles were classified as previously described (see chapter 2, pages 23-25) and every section was examined for healthy and atretic large follicles, i.e. those classified as stage V, VIa & VIb. The criteria for identifying atresia have been described in chapter 2 (pages, 27-28).

Table 4.1:

Number of animals (CBA/Ca mice) in each treatment group i.e. progesterone implant inserted (implant group) or empty implant inserted (sham group). Age is that at time of sacrifice. All animals were operated when aged between 54-58 days post partum.

Age range in days	Treatment time in days	Numbers of animals	
		Implant group	Sham group
60-70	2-20	8	2
71-91	21-40	21	4
92-138	41-80	5	3
140-175	80-120	6	3

Analysis of data:

The mean +/- S.E.M. was calculated for each follicle stage for four age groups (see table 4.1) in control and experimental group, and differences were analysed using one-way analysis of variance to test for statistical significance. Data have been expressed in terms of the number of days treatment; atresia data has been expressed as the mean number of atretic follicles and percentage of atretic follicles at stages, V, VIa & VIb. Smaller stages of follicles were not scored for atresia because morphological signs were difficult to detect at these stages.

Mathematical modelling:

Compartmental analysis was applied to those data falling within the age span of 100 days so as a direct comparison could be made with the earlier model for "control" animals. Non-parametric regression analysis to guide the fitting of the parametric model was applied (Faddy & Jones, 1987). Parameter estimates to describe follicle growth and death through 5 stages of development were computed and "constrained" parameter estimates were also obtained. The constrained estimates applied the same growth rates as those formulated for "normal" CBA/Ca animals.

Results:

The results obtained from the preliminary study showed that normal vaginal smear patterns were abolished in both treatment groups within 2 days of insertion of the implant, with vaginal smears showing a dioestrous pattern. Animals in the control group continued to present normal oestrous cycles. Neither oviductal oocytes nor corpora lutea

were found in the progesterone implant treatment groups, but these were observed in all but one of the control group. Figure 4.1 shows the results of the progesterone assay from these three groups. Plasma levels of progesterone in those animals with one implant were increased by a factor of 2 over the controls and the levels in those with two implants by a factor of 4. These results were statistically significant ($p < 0.01$).

Figures 4.2a-e shows the mean follicle counts for five stages of follicular development in control and progesterone implant group for four time intervals (implants in place for 2-20; 21-40; 41-80 and 81-120 days). No statistically significant differences were found in the number of stage I follicles between the treated and the untreated group (Figure 4.2a). Although the numbers of stage I follicles at days 21-40 days after treatment are just on the boundary of being significant. This may indicate differences in the rate of loss of stage I follicles with this being more rapid in the treated group but there were no differences as treatment time progressed.

The mean numbers of stage II follicles in the progesterone treated group were found to be significantly lower ($p < 0.01$) than the comparable controls after 2-20 and 21-40 days implant (Fig. 4.2b). This difference diminished as time progressed, and the differences in numbers of follicles at stage II after 41-80 and 81-120 days in the two groups were not significant at the 5% level with $p = 0.058$ and 0.053 respectively.

Figure 4.1:

Mean (+ S.E.M) serum levels of progesterone in control animals and animals with one or two progesterone implants in place for 16 days.

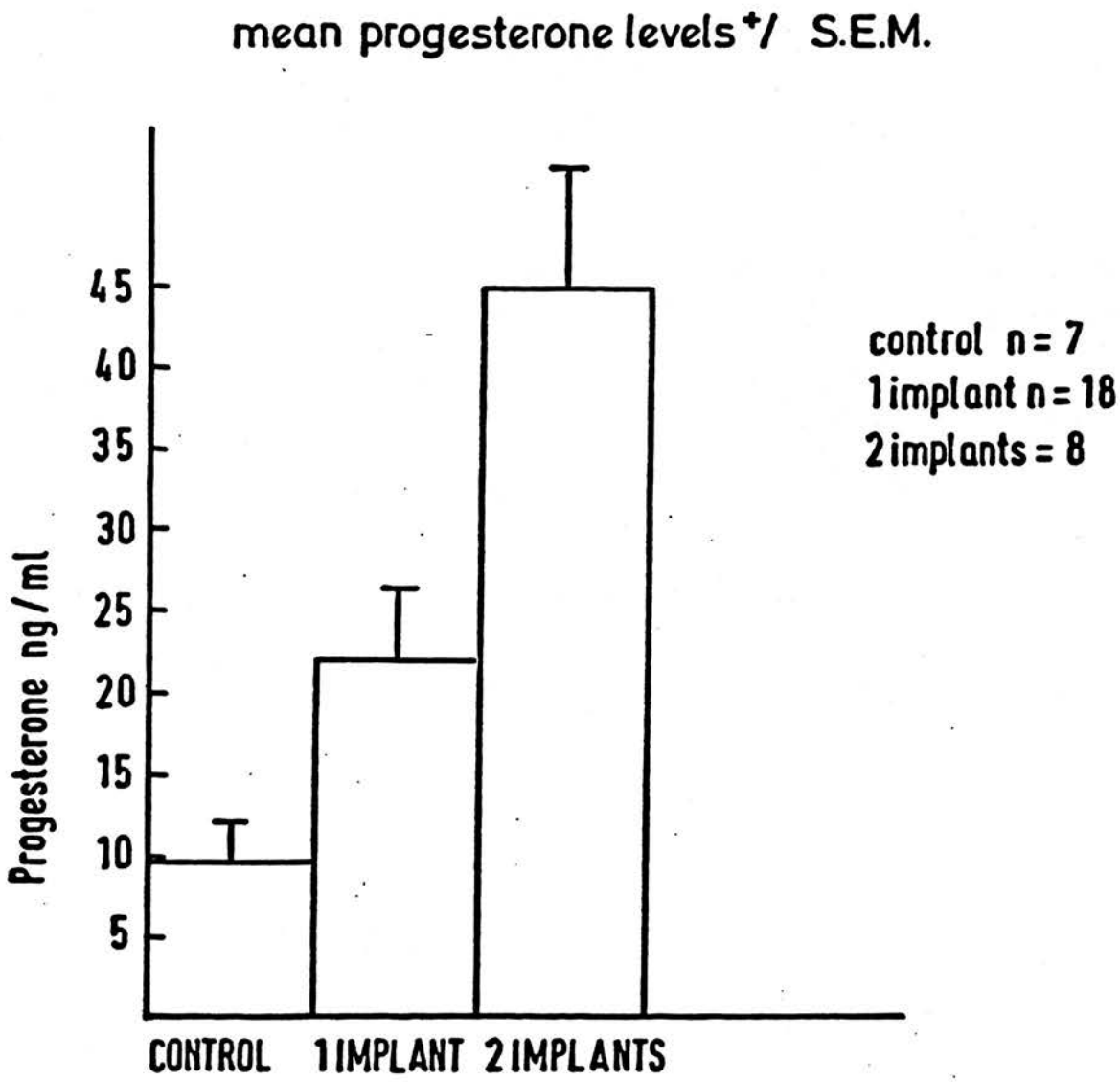


Figure 4.2 a-e:

These 5 histograms represent the mean (+S.E.M) number of follicles at stages I-V+ in control and progesterone treated animals over four treatment times. Number of animals in each group (n)=

2-20 days control n=2	2-20 days progesterone n=8
21-40 days control n=4	21-40 days progesterone n=21
41-80 days control n=3	41-80 days progesterone n=5
81-120 days control n=4	81-120 days progesterone n=6

Figure 4.2e shows mean (+S.E.M) number of atretic as well as healthy stage V+ follicles

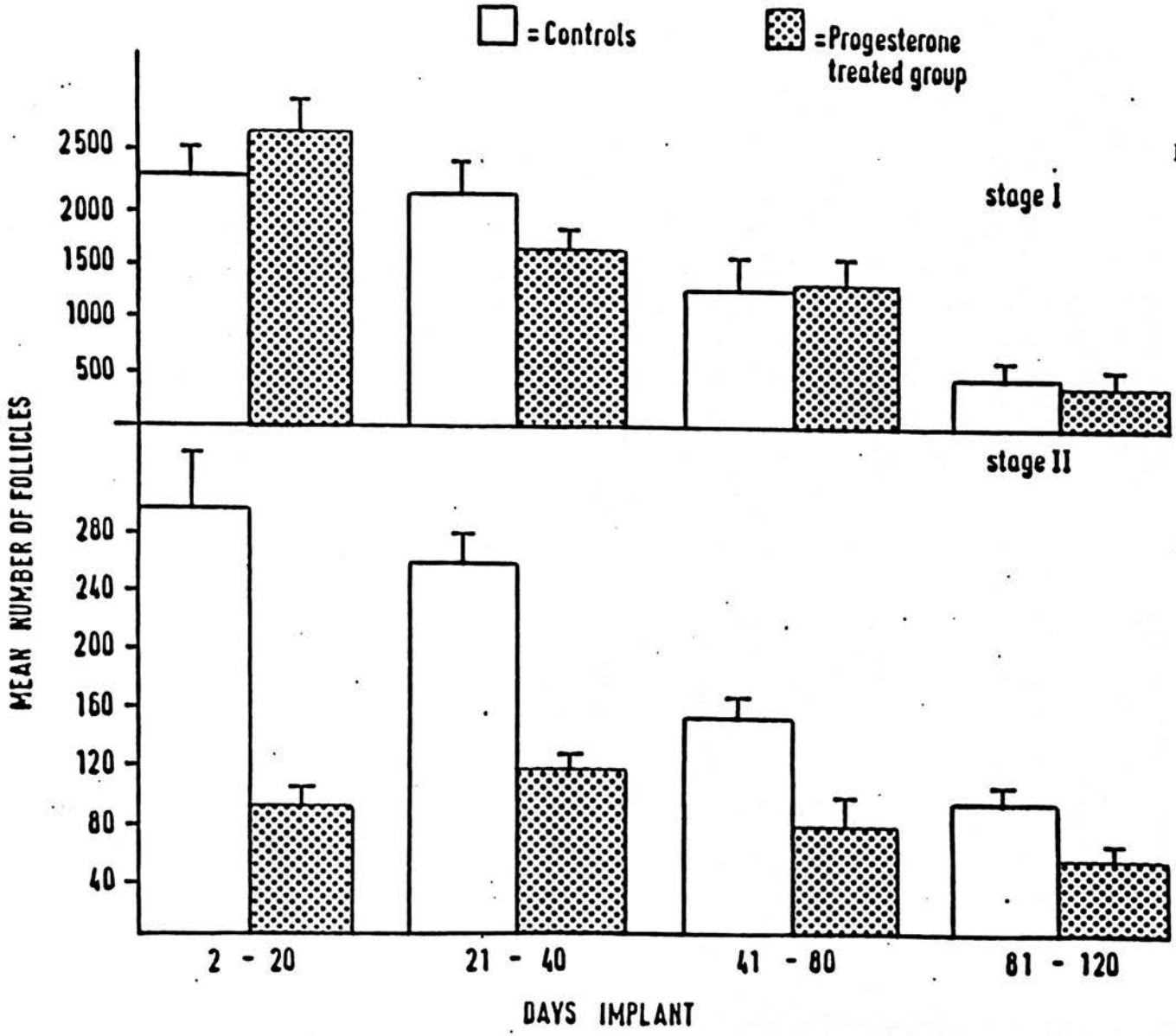


Fig. 4.2a

Fig. 4.2b

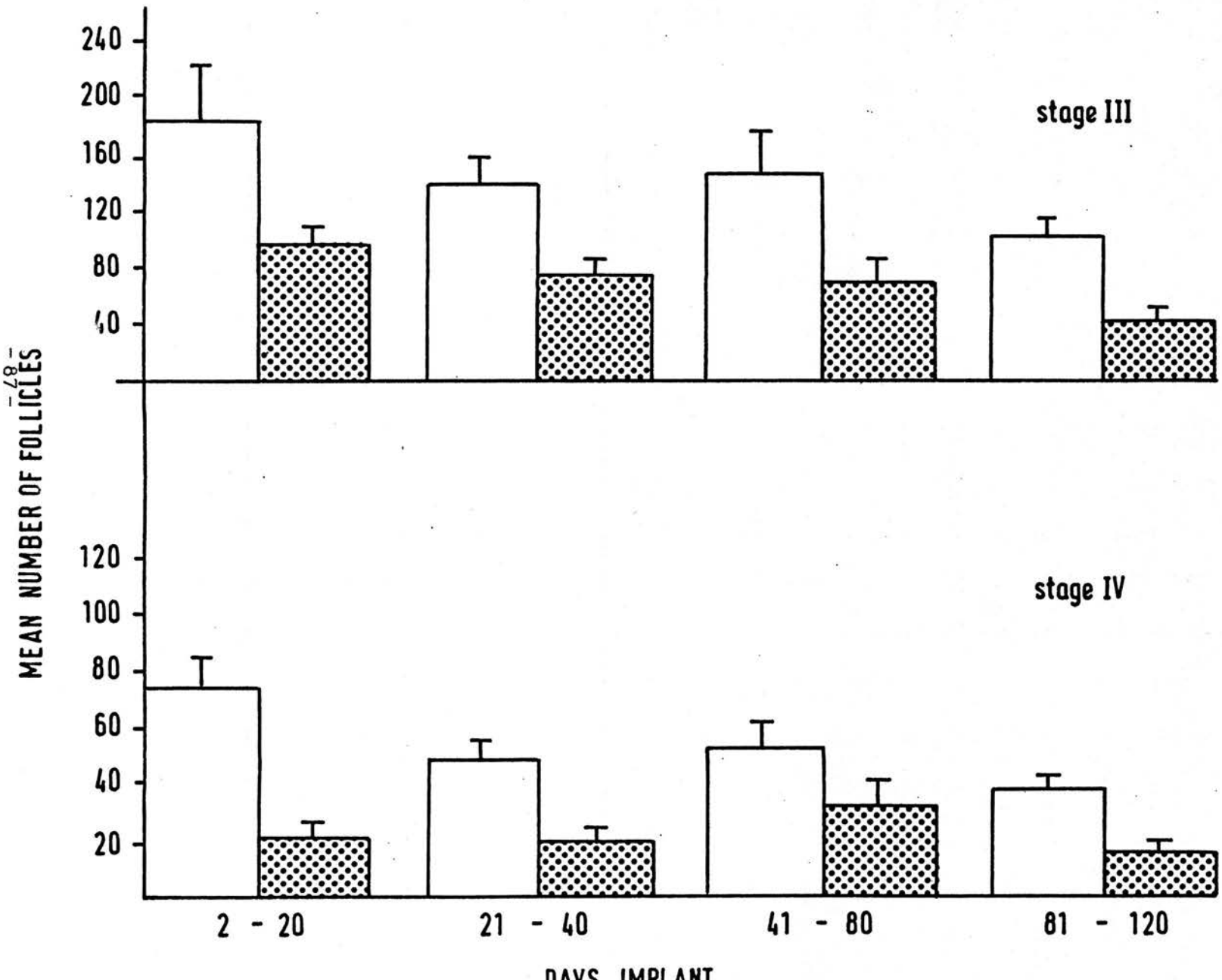


Fig. 4.2c

Fig. 4.2d

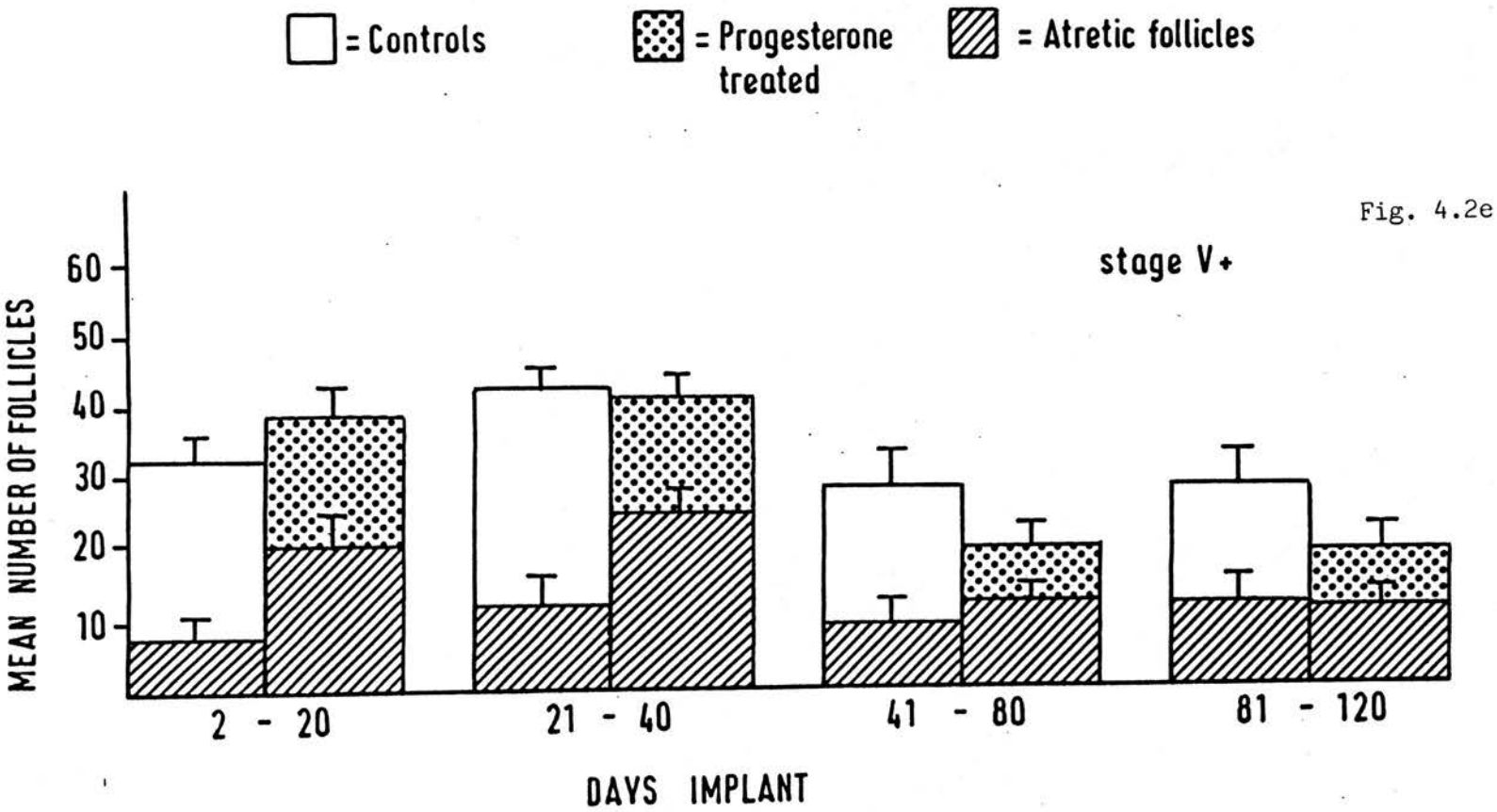

= Controls
= Progesterone treated
= Atretic follicles
stage V+
MEAN NUMBER OF FOLLICLES
60
50
40
30
20
10
2 - 20
21 - 40
41 - 80
81 - 120
DAYS IMPLANT

Fig. 4.2e

Numbers of stage III and IV follicles were found to be significantly lower in the treatment group during the 2-20 (p=.03 & <0.01) and 21-40 (p= <0.01) day time interval. Stage III follicles were significantly lower in the treatment group after 41-80 (p<0.01) and 81-120 (p<0.01) days after treatment but no statistically significant differences were found in the numbers of stage IV follicles at these times (Fig. 4.2c,d).

No statistically significant differences were found between the number of stage V+ follicles in treated and control animals at all time intervals (Fig. 4.2e). Figure 4.2e also illustrates the number of stage V+ follicles that had signs of atresia. The number of atretic stage V+ follicles were significantly greater in the treatment group after 2-20 and 21-40 days of treatment (p<0.01). There were no significant differences in numbers of atretic follicles in both groups after 41-80 and 81-120 days treatment.

The distribution of normal and atretic follicles between the sub-divisions of stage V+, i.e. stages V, VIa & VIb is shown in figure 4.3. The numbers of follicles in each of these stages were similar in both groups at all four time intervals with the exception of the number of stage V follicles in treated group after 2-20 days implant which were significantly greater than controls (p <0.01). The number of stage, V, VIa & VIb with signs of atresia was significantly greater in the treatment group after 2-20 days and 21-40 days of treatment (p<0.01). No significant differences were found in the number of atretic follicles at all stages after 41-80 and 81-120 days after treatment (Fig. 4.3).

Figure 4.3:

Mean number (+S.E.M) of healthy and atretic stage V, VIa & VIb follicles in control and progesterone treated animals over four treatment times.

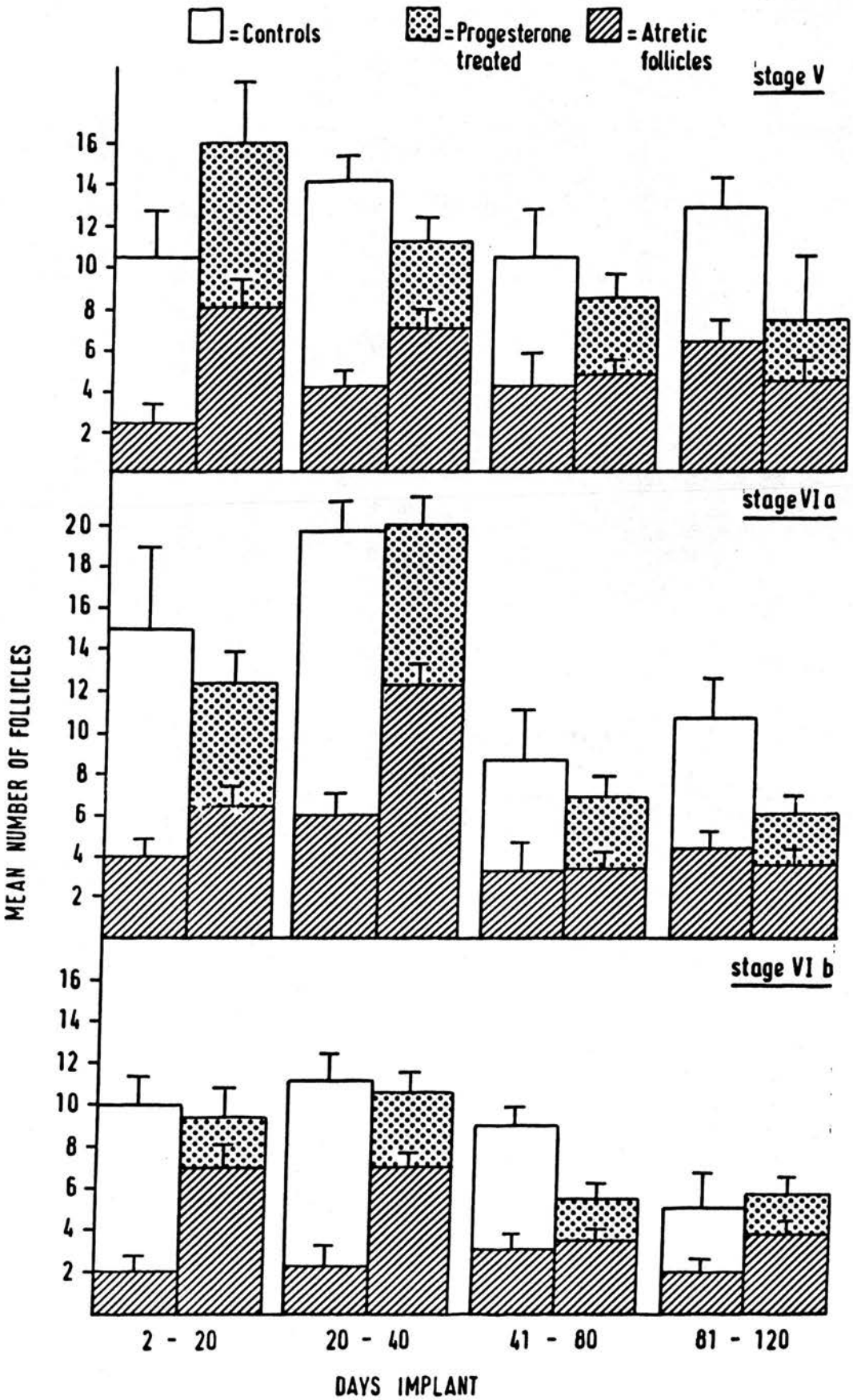

= Controls
= Progesterone treated
= Atretic follicles
stage V
stage VI a
stage VI b
MEAN NUMBER OF FOLLICLES
2 - 20
20 - 40
41 - 80
81 - 120
DAYS IMPLANT

Figure 4.4:

Mean (+S.E.M) percentage of stage V, VIa & VIb follicles showing signs of atresia in control and progesterone treated animals over four treatment times.

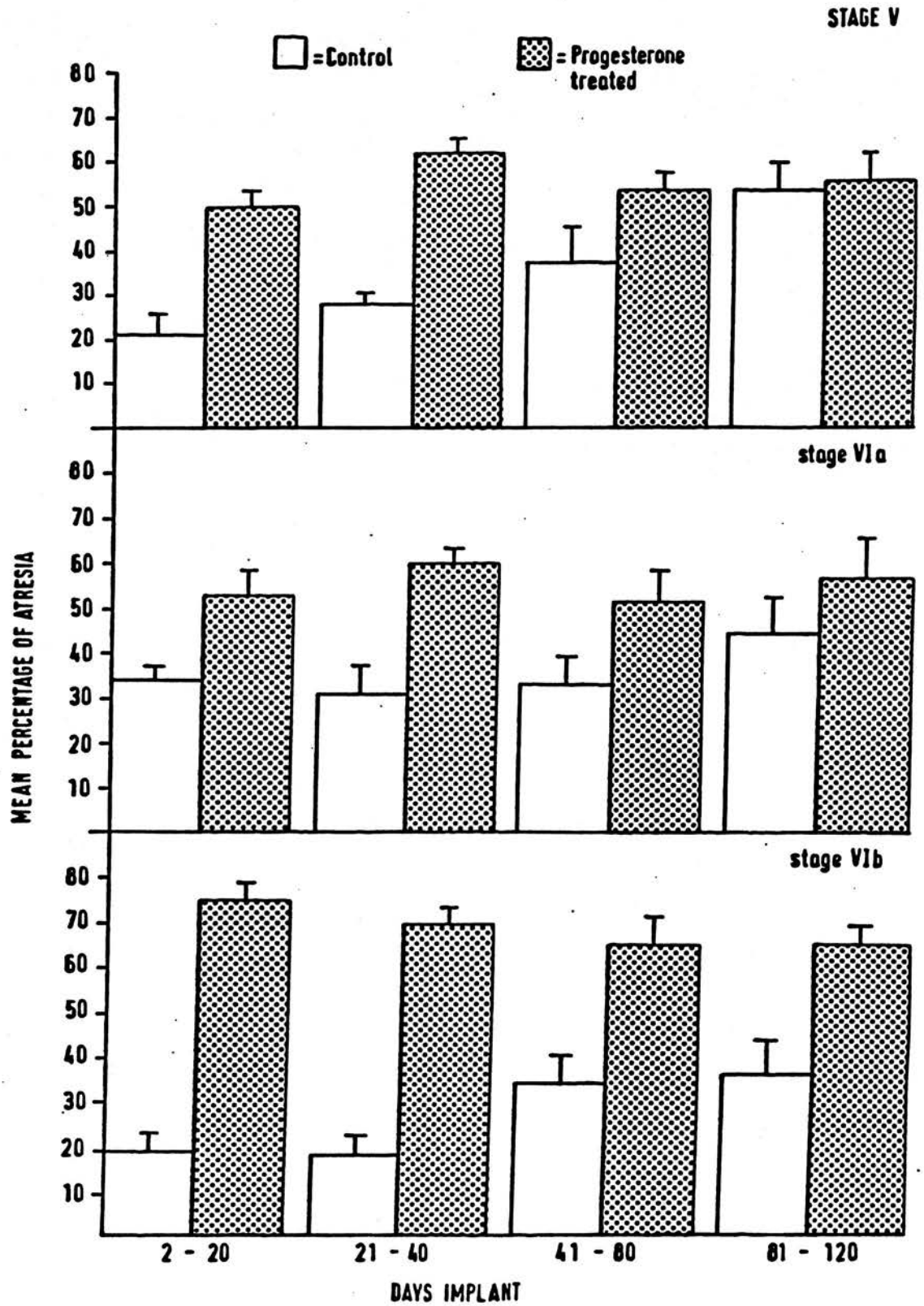

STAGE V
= Control
= Progesterone treated
stage VIa
stage VIb
MEAN PERCENTAGE OF ATRESIA
80
70
60
50
40
30
20
10
2 - 20
21 - 40
41 - 80
81 - 120
DAYS IMPLANT

Figure 4.4 shows the same data as in figure 4.3 but expressed in terms of the percentage of atretic stage V, VIa & VIb follicles in control and treatment groups at 4 time intervals. There were significant differences in this percentage value between control and treated groups after 2-20 and 21-40 days. The difference in incidence of atresia between control and treatment groups decreased with time because of an increase in the proportion of atretic follicles at these stages in the control group (41-80 days and 81-120 days).

Compartmental Modelling:

Figure 4.5a-e shows the results of follicle counts obtained from the ovaries of those animals treated with a progesterone for between 2-40 days. The data are illustrated graphically with the animals age given as the independent variable in order to directly compare the distribution of follicles with that of the normal animals (chapter 2). Parametric and non-parametric regression lines which were derived from the modelling are shown on figure 4.5a-e and both lines correspond closely also superimposed upon these curves are those curves derived for "normal" CBA/Ca animals (see pages 33-34). The curves obtained from the progesterone treated group deviates significantly from the normals. The parameter estimates describing this fit are shown in figure 4.6(a) and no time dependent changes are evident during this age span. Also shown in this figure (b) are the parameter estimates obtained if it is assumed that growth rates in the treated group are equivalent to those estimated for "normal" CBA/Ca mice of comparable ages (see page, 37). These models will now be discussed and compared.

Figure 4.5a-e:

Graphs a-e (pages 93 & 94) represent the number of follicles (stages I-V$^+$) found in each of 29 animals treated with progesterone aged 60-91 days. Progesterone implants were inserted when animals were aged 55-58 days. The data were obtained by differential counting of follicle stages.

The dotted line corresponds to the fit of the parametric regression obtained from the application of compartmental modelling to the data. The dashed line corresponds to the non-parametric regression and the bold unbroken line represents the parametric curve describing follicular dynamics of "normal" CBa/Ca mice from birth to 98 days (see chapter 2)

Fig. 4.5a

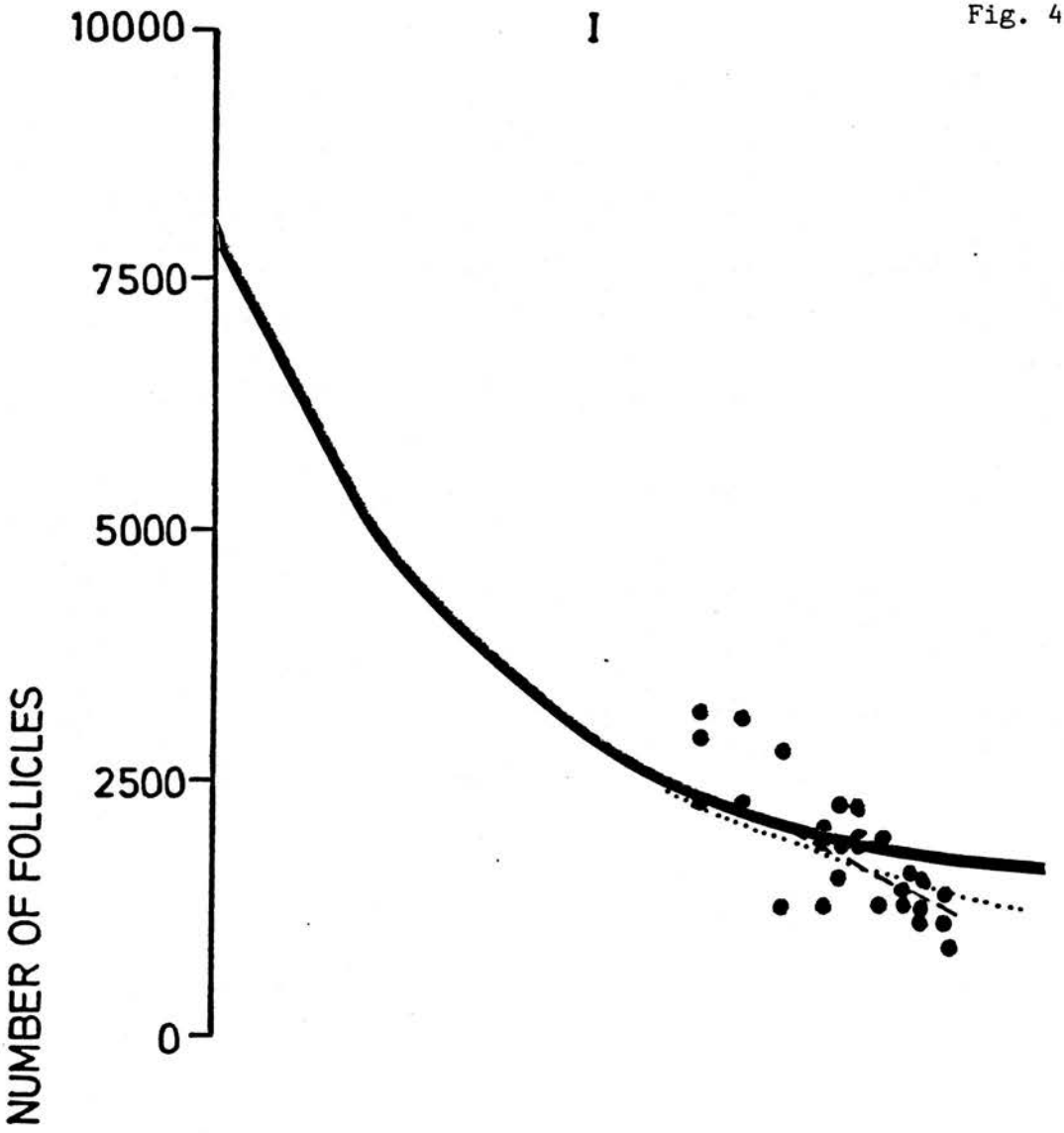


Fig. 4.5b

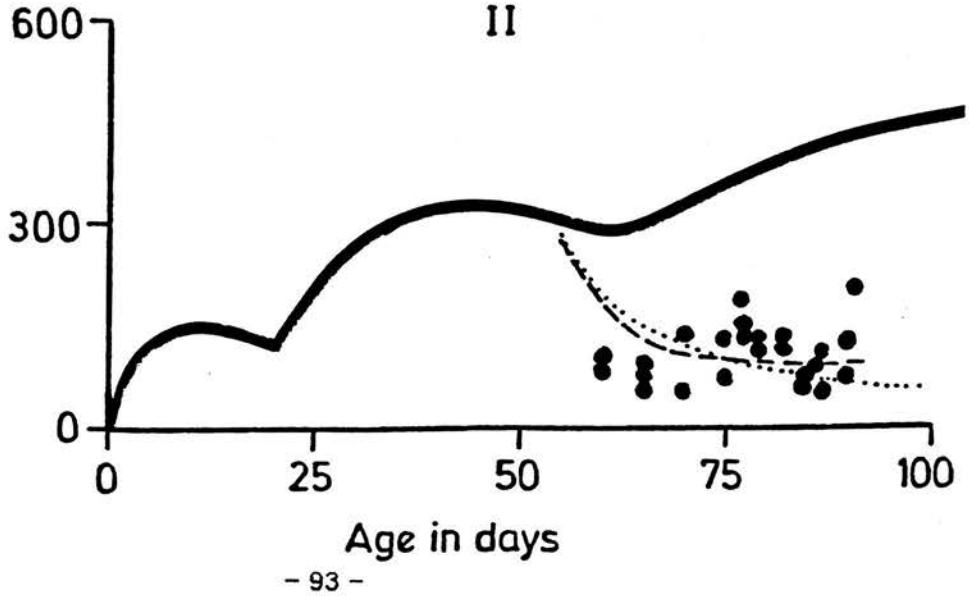


Fig. 4.5c

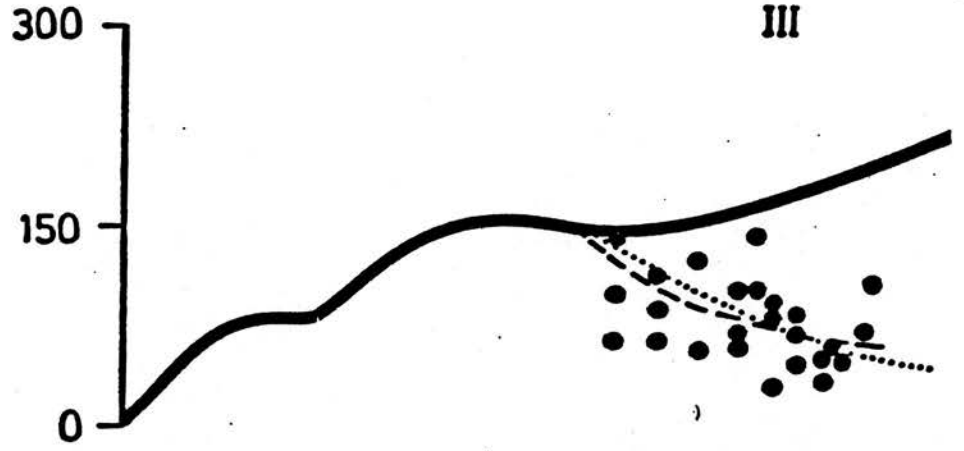
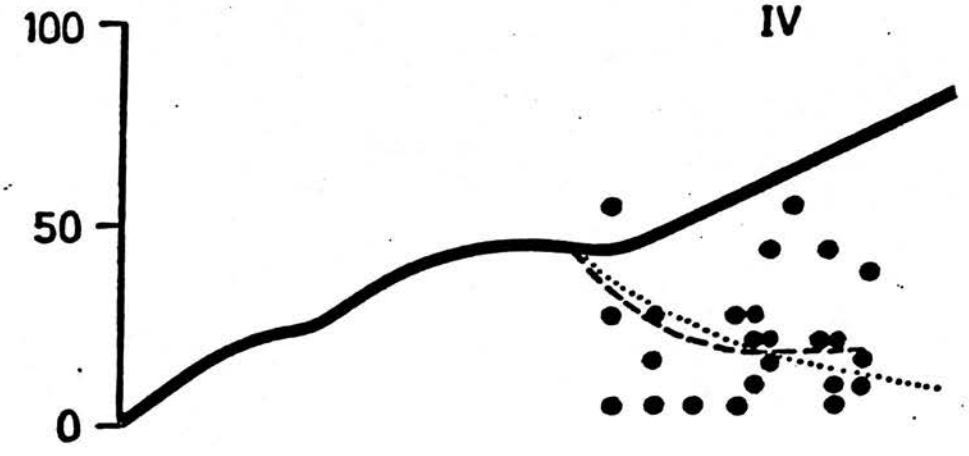


Fig. 4.5d



NUMBER OF FOLLICLES

Fig. 4.5e

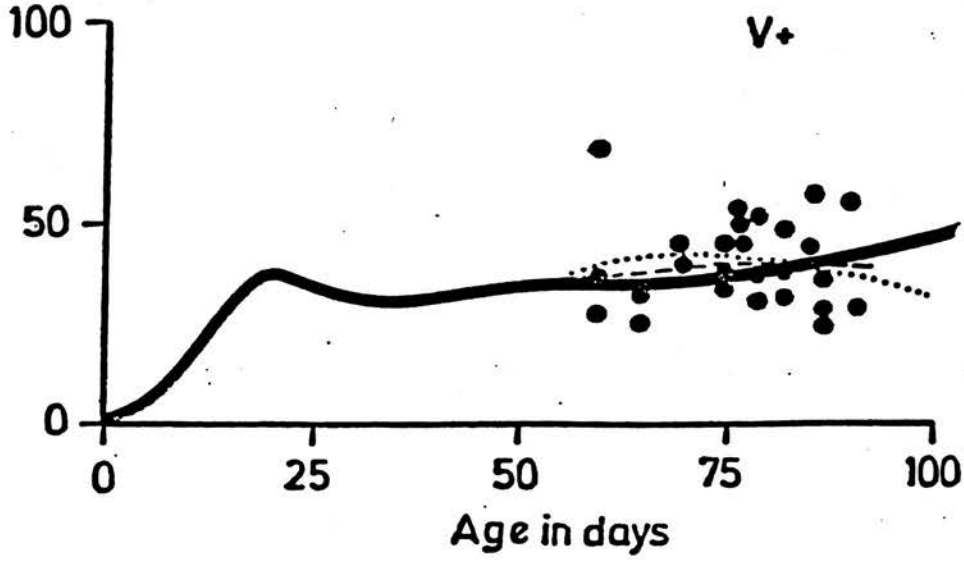


Figure 4.6:
Parameter estimates obtained for growth (migration) and death (atresia) through five stages of follicular development in progesterone treated animals. a)= unconstrained parameters. b)= constrained parameters i.e. growth rates n same as in controls (chapter 2).

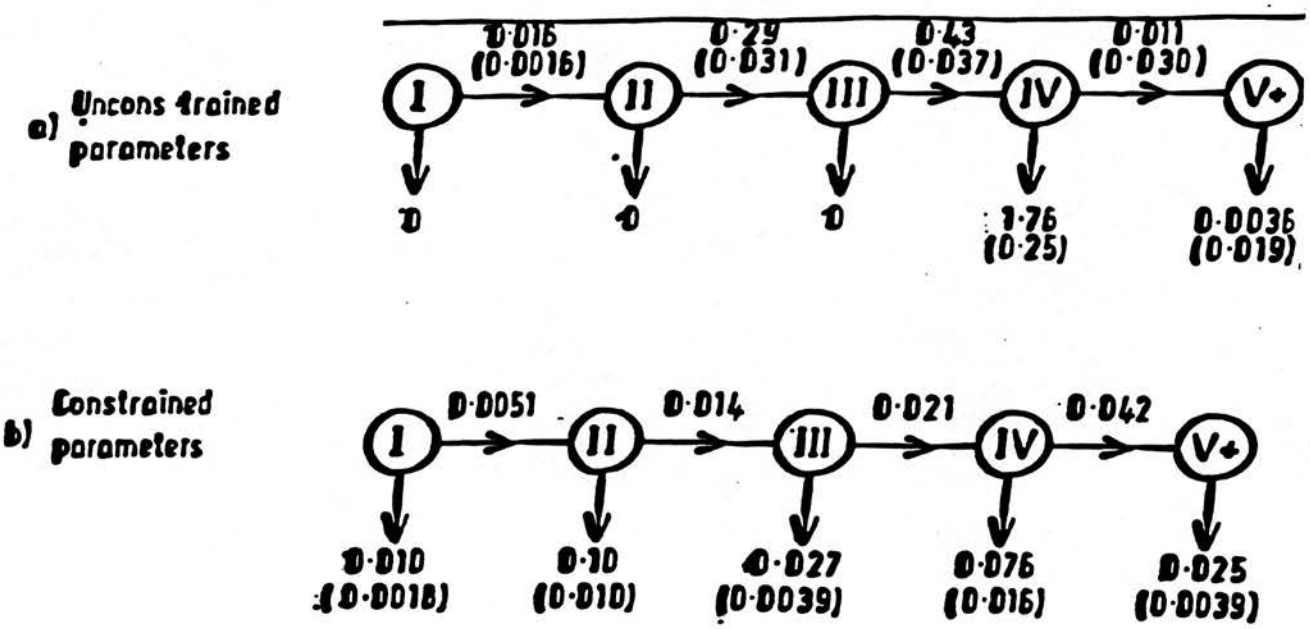


Table 4.2:
Average numbers of follicles leaving the stages (compartments) per day, either by growth to the next stage or death. Estimated from the unconstrained model.

Age	I→	→II	II→	→III	III→	→IV	IV→	→V+	V+→
63	35	35	42	42	48	48	50	0.3	0.1
70	31	31	34	34	36	36	37	0.2	0.1
77	28	28	30	30	31	31	32	0.2	0.2
84	25	25	27	27	28	28	28	0.2	0.2
91	22	22	24	24	25	25	25	0.2	0.2
98	20	20	21	21	22	22	22	0.1	0.2

The unconstrained parameter estimates (figure 4.6a) indicate that deaths from the earlier stages of follicular development, (I-III) were low in the progesterone treated group. If these estimates are compared with those obtained for the normal animals of a similar age group the results indicate that the total rate of loss from the pool of stage I follicles is significantly greater ($p<0.025$) than that obtained for the normal group. The rate of movement from stage II-III is increased substantially in the progesterone treated animals compared with the normals as is the rate of movement from stage III-IV. Greater differences emerge at stage IV as the parameter estimates indicate that there is a fast rate of movement from stage IV with a large proportion of deaths at this stage. These conclusions are evident even without modelling when the falling numbers of stages II-IV are considered against the controls (see figures 4.2a-e).

The average number of follicles leaving each stage per day is shown in table 4.2. These values have been estimated from the model using the unconstrained parameter estimates and the values obtained indicate a high rate of movement through the follicle stages. More follicles move through follicle stages II-IV per unit time compared with the values obtained for the normal animals (see page, 39). Since deaths at stages I-III are estimated to be low, all of the follicles leaving one compartment must enter the next. The numbers of follicles entering the final stages of follicular development appear to be modulated, since of those leaving stage IV only a small number develop to stage V (approximately 0.2 follicles per day) (table 4.2) compared with the estimate of 2 follicles per day in control animals. Thus growth into stage V+ has been substantially reduced in comparison with the

parameter estimates obtained for normal animals aged 60-100 days and the rate of movement out of stage V+ significantly slower than that observed for the normals.

Figure 4.6b shows the constrained parameter estimates obtained if it is assumed that the treatment should not alter the rate of movement through follicle stages. The parameter estimates for migration through the stages are constrained to follow the normal estimates. The resultant estimates indicate a distribution of death at the earlier stages of follicular development and in particular massive deaths at stage II of follicular development. These estimates have not been used since there is no evidence to support the assumption that treatment would not alter "migration" rates through the stages

The numbers of stage V+ follicles were similar in both groups and differences between the treatment and control animals may be occuring within this group. The model is unable to provide reliable indications of rates of movement within this group (i.e. between V, VIa & VIb) since the numbers concerned are low. An estimation of the number of healthy and atretic follicles at each of these groups was obtained using morphological criteria to determine some of the changes that might be occurring.

Figure 4.7 gives the mean number of healthy and atretic follicles at stages V, VIa and VIb in three groups of animals aged 60-100 days. The normal controls are those animals from previous study and are shown here to demonstrate that the results for sham operated controls and the other control group were virtually identical. The normal controls

were used in this instance since the follicle distribution is being studied in relation to age rather than length of implant period as in the earlier analyses of atresia.

The mean number of large follicles found in each of the three stages were not significantly different in control and treated animals. The greatest number of follicles were stage VIa with the least being stage VIb. The number of atretic follicles at each stage was significantly greater in the progesterone treated group ($p<0.025$) with the numbers in sham and normal controls not being significantly different.

In order to highlight the contribution that atretic follicles make to these stages of follicle development the data were expressed in terms of the percentage of atretic stage V, VIa and VIb follicles in treatment and control groups (figure 4.8). Almost 60% of stage V and VIa follicles were atretic in the progesterone treated group compared with 30-40% in the control groups at similar stages. 70% of stage VIb follicles in the progesterone treated group were atretic compared with around 20% in the control groups. The percentage of atretic follicles decreases at stage VIb in the control groups but no decrease is shown in the treated group.

Figure 4.7:

The histograms represent the mean (+S.E.M) number of histologically normal and atretic stage V, VIa & VIb follicles found in control and progesterone treated animals aged 60-91 days.

Normal controls= animals within this age group from previous study (chapter 2) n=20

Sham controls= control animals with empty implant inserted n=9

Progesterone treatment group n=29.

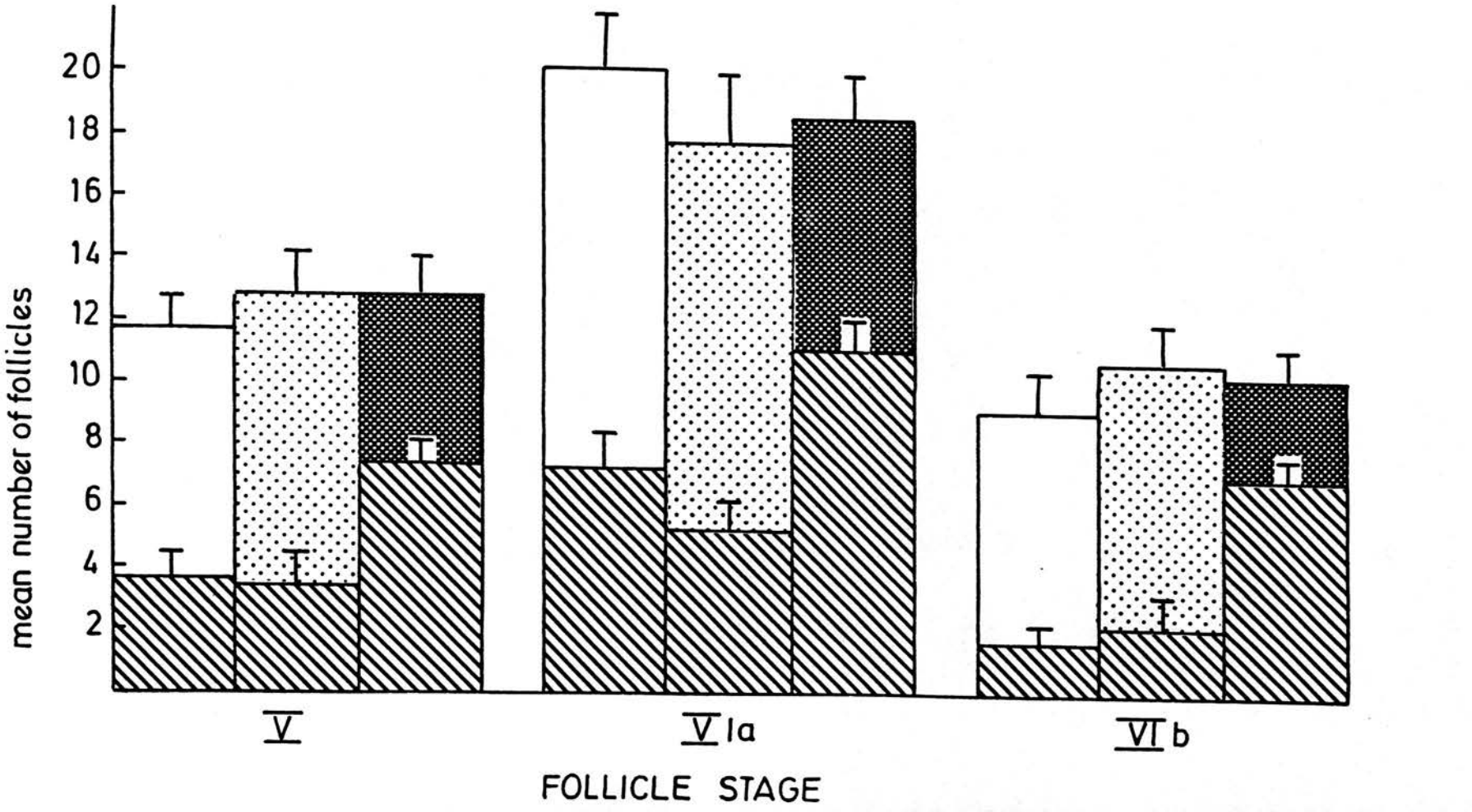
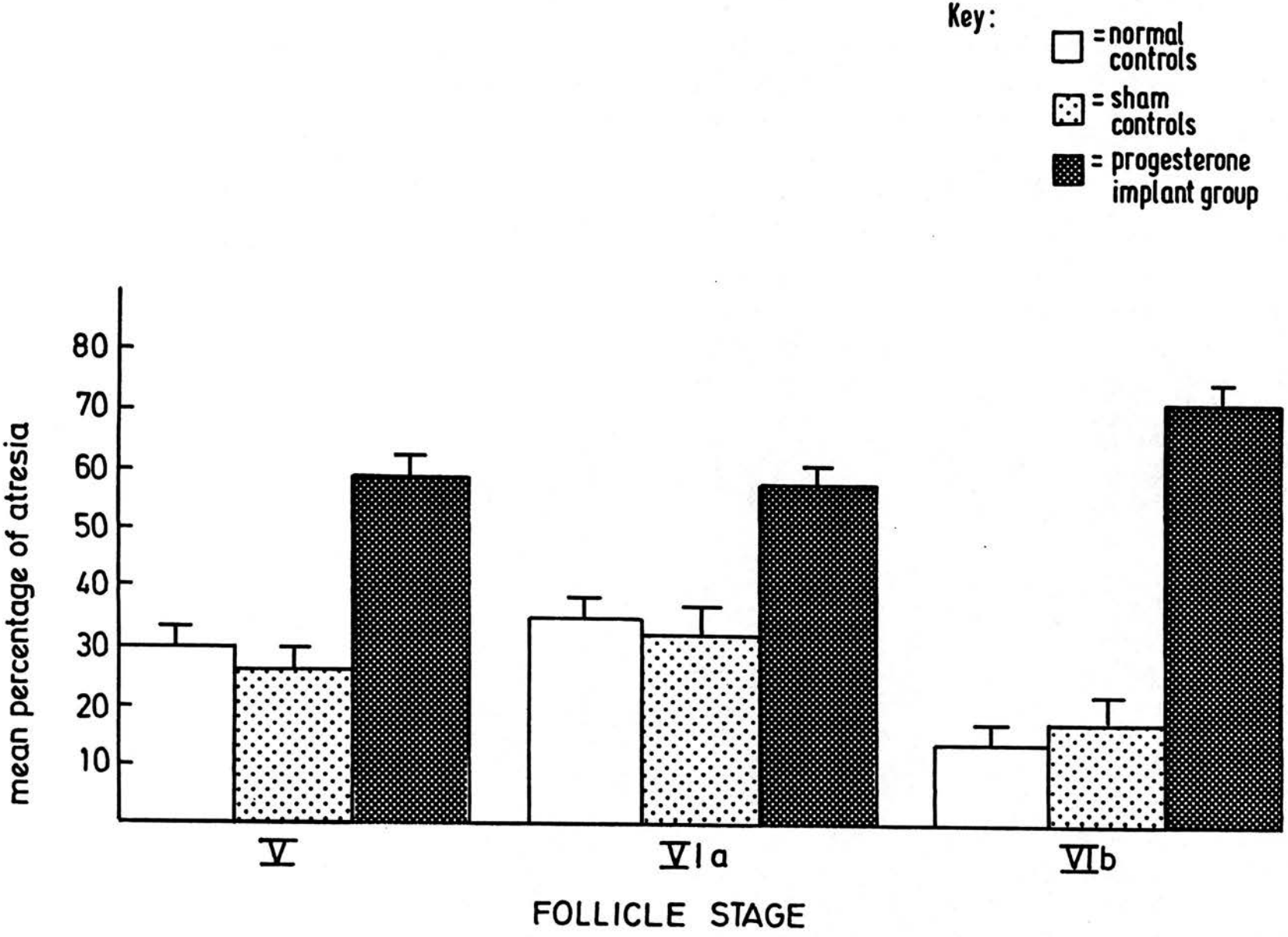

Key :
= normal controls
= sham controls
= progesterone implant group
atretic follicles
mean number of follicles
20
18
16
14
12
10
8
6
4
2
V
V Ia
VI b
FOLLICLE STAGE

Figure 4.8:

The histograms represent the mean (+ S.E.M) percentage of stage, V, VIa & VIb follicles with signs of atresia in control and progesterone treated animals aged 60-91 days.


Key:
= normal controls
= sham controls
= progesterone implant group
mean percentage of atresia
80
70
60
50
40
30
20
10
V
VIa
VIb
FOLLICLE STAGE

Discussion:

Despite considerable research, the effects of progesterone on follicular development are unclear. The problem is compounded by the probable species differences that exist. Studies carried out on the pregnant rat have shown that only small preantral follicles are present throughout both pregnancy and pseudopregnancy; only at the end of pregnancy do large follicles emerge (Greenwald, 1966). In the monkey follicle growth is slow throughout pregnancy and Graafian stages are suppressed (diZerega & Hodgen, 1979). Evidence of inhibitory effects of exogenous progesterone on follicular development have been obtained in a number of species, viz. rats (Buffler & Roser, 1974) hamsters, (Greenwald, 1977) and rhesus monkey (Goodman & Hodgen, 1977) but it does not neccessarily follow that this is the hormone responsible for effects on follicles during pregnancy.

High levels of progesterone inhibit ovulation (Hoffman & Schwartz, 1965; Richards, 1980) and one mechanism by which progesterone may inhibit the development of ovulatory follicles is through negative feedback effects on gonadotrophin release via its action on the hypothalamic-pituitary axis (Greenwald, 1978; Taya *et al.*, 1981; Garza & Terranova, 1984). There are, however, paradoxical effects of this hormone as progesterone can advance the time of ovulation in the 5-day cycling rat by facilitating the release of LH on dioestrus to result in a 4-day cycle (Everett, 1948; Zeilmaker, 1966). The suppression of follicular development in the pregnant animal has been partly explained by the inhibitory action of progesterone on circulating gonadotrophin levels (DePaolo & Barraclough, 1979). An in vivo study in sheep has shown that progesterone decreases serum levels of FSH (Goodman, *et al.*, 1981) and

in vitro studies on ovine pituitary tissue indicates that progesterone has a direct inhibitory effect on FSH secretion (Batra & Miller, 1985), other studies in this species have shown no effect (Moss *et al.*, 1981; Clarke & Cummins 1984).

There is experimental evidence which suggests that progesterone may also have direct effects on follicular development (Moore & Greenwald, 1974; Schrieber et al, 1980;). Progesterone receptors have been found in the ovaries of the rabbit and the rat providing a basis for hormone action (Philibert, et al, 1977; Schreiber & Hsueh, 1979). Inhibition of ovulatory follicles in rats has been found when progesterone was given prior to or in conjunction with gonadotrophins (Fukuda *et al.*, 1980) and there is some evidence from in vitro studies that progesterone acts on granulosa cells to inhibit the production of oestradiol thus creating an environment which predisposes the follicle to atresia rather than ovulation (Fortune & Vincent, 1983). A study on the rabbit has shown that elevated progesterone levels do not affect LH or FSH levels and therefore all effects on the follicular system are a direct result of progesterone acting within the ovary (Setty & Mills, 1987).

Most of the studies looking at the effects of elevated progesterone on follicular development have concentrated on the larger follicles and little attention has been paid to earlier developmental stages. This study was designed to look at the effect of blocking ovulation by progesterone on follicles at all stages of development and to determine the dynamics of follicle growth by the application of mathematical modelling in those animals from 55 to 100 days old. Silastic implants of progesterone were effective at blocking ovulation, a single implant

producing 22 ng/ml of progesterone in serum. This level is within the range of progesterone levels reached during mid pregnancy in mice (Murr *et al.*, 1974b; McCormack & Greenwald, 1974; Virgo & Bellsard, 1974) although the hormonal conditions in this experiment differ substantially in other respects from the pregnant animal because placenta and normal functioning corpora lutea are absent.

The data obtained from this study were analysed using compartmental modelling but this was done only for data obtained from animals aged between 55-100 days (i.e. 2-45 days of treatment), since these could be directly compared with the parameters obtained for normal animals (see chapter 2). The compartmental model has demonstrated that the dynamics of preantral follicle development in progesterone-treated animals differ from those of cycling animals. Two sets of parameter estimates have been presented in the results to demonstrate two possible mechanisms by which the profile of follicle stages obtained could have arisen. One set has been constrained by the assumption that the rate of follicle growth is unaffected by treatment since studies during pregnancy indicate that the rate of growth of follicles remains unchanged (Pedersen & Peters, 1971), it is, however difficult to make direct comparisons between the pregnant state and steroid treated animals since the treated animals have had a continuous exposure to increased levels of progesterone ranging from 2-45 days in the short term treatment group and upto 140 days in the longer term treatment group. The constrained model indicates that the follicle profile observed must have arisen through an increase in deaths at intermediate stages of follicular development. Deaths at the intermediate stages were not observed histologically, but these are difficult to detect. There is,

however, no substantial evidence to assume that growth rates would remain unchanged in the treatment groups therefore only the unconstrained parameter estimates will be discussed.

In this study the elevated levels of serum progesterone affected the growth and death rates at all stages of follicular development. A greater rate of loss than normal from the primordial pool in the progesterone treated group up to 100 days of age was inferred. This effect may be reduced with time since the mean numbers of primordial follicles in animals with longer term implants were not significantly different from controls. These results contrast with results obtained from studies on follicle dynamics in the mouse during pregnancy (Pedersen & Peters, 1971) using autoradiographs of ovaries after pulse labelling with tritiated thymidine, it was concluded that fewer follicles began to develop per unit time during pregnancy but those starting their growth phase developed at the same speed as follicles in the non-pregnant animal. If the rate of depletion from stage I estimated by the model for the first 100 days continued the ovary would soon be depleted of follicles. Results of follicle counts from the long term treatment group indicate that there is a slowing of the rate of depletion from stage I therefore some modulation must be occurring as treatment progresses. These results have implications for the effect of oral contraceptives on the follicular population both in the long and short term. If by blocking ovulation the dynamics of follicle growth are affected then it may be expected that age of menopause may be influenced by oral contraceptive history and parity in the human. There does not seem to be any evidence to suggest that either of these factors affects the age at which menopause occurs (Gosden, 1985b). The

results obtained from this study do not support long term differences in the rate of depletion of the primordial pool.

The mean number of follicles at growing II, III & IV were significantly lower in short and long term progesterone-treated animals but the numbers of stage V+ follicles were similar in both groups up to 40 days of treatment. If stage I follicles begin to grow at a greater rate than that for control animals (or even at the same rate as may be the case in the longer term group) the observed decline in the numbers of small growing follicles in the treatment group must have resulted from an increased rate of movement through successive stages of follicle development or alternately by follicle deaths at each of these stages. The parameter estimates generated by the application of compartmental modelling indicate an increased rate of movement through the follicle stages. There is some evidence in the rabbit that there is a greater accumulation of small follicles during pregnancy and progesterone implant treatment (Setty & Mills, 1987).

Enhanced follicle growth at early stages of development need not be under the control of gonadotrophins (Faddy *et al.*, 1983; Halpin *et al.*, 1986) and a direct effect of progesterone may be the cause of the increased growth of preantral follicles. A stimulatory effect of progesterone on follicular growth has been observed and results from in vitro studies have shown that there is an increased uptake of ^{3}H-thymidine in cultured ovarian cells from immature mice after the addition of progesterone (Kent, 1973). A stimulatory action of progesterone on small follicles, has been found in studies using hypophysectomised animals. The number of follicles recruited by

gonadotrophin treatment in hypophysectomised animals was significantly increased if a high dose of progesterone was administered (Kim & Greenwald, 1987), and these workers suggest that at high doses (1mg) progesterone acts as a mitogenic stimulus on granulosa cells. At lower doses overall number of large follicles was not affected but the number of atretic follicles was significantly increased (Kim & Greenwald, 1987). It has also been shown that progesterone can enhance its own production in vitro (Goff *et al.*, 1979; Fanjul *et al.*, 1983) thus its effects may be enhanced in vivo.

Whilst follicles are growing quickly through the smaller stages an increase in deaths at stage IV is indicated by the model. Why should follicle growth increase initially only to lose many follicles at later stages? The function of the ovary is to provide a constant supply of fertile oocytes thus if large follicles are unable to undergo the final maturational changes to ovulatory follicles, as in the treatment group, feed-back mechanisms may operate on small follicle stages leading to an increased supply of follicles which can be used if the correct conditions prevail.

Although ovulation was blocked, follicles still proceeded to antral stages of development although there a slower rate of movement of follicles to stage V compared with the control group was estimated. A problem arises when comparing the number of large follicles in the control group and the treatment group since there is no direct compatability between the stage of oestrous cycle and the acyclic animal. Antral follicles undergo marked changes during the ovarian cycle (Hirshfield, 1986b) and the percentage of atretic follicles varies

during the stages of the cycle (Numazawa, & Kawashima, 1982). The choice of pro-oestrous was made on the basis that it can be identified unequivocally on the basis of vaginal smears and can indicate the numbers of ovulations that are imminent.

The numbers of stage V+ follicles found in the short term implant group were similar to controls, but were reduced in the long term implant group. Stage V+ is a heterogeneous group consisting of three subdivisions ranging from large preantral to large antral follicles. It might be expected that the distribution of follicles between these subdivisions would differ in the control and implant treated groups if gonadotrophins were low because antral follicle growth would be impared. This expectation was not found. The number of stage V, VIa and VIb follicles was similar in treatment and control groups.

High levels of progesterone as already indicated, may enhance the response of small antral follicles to subtle increases in LH (Richards & Bogovich, 1982) thus enabling the growth of antral follicles during pregnancy. In studies on the pregnant rat, antral follicles were found throughout pregnancy (Richards & Kersey, 1979) but in a study of the pregnant mouse no antral follicles were found between days 10 and 14 of pregnancy (Greenwald & Choudary, 1969). During early pregnancy the antral follicles found were capable of ovulating in response to exogenous hCG (Richards & Kersey, 1979) but in later stages of pregnancy they were not (Greenwald, 1966). In mice, follicles that ovulate after the litter is born must have started their growth at the onset of pregnancy (Pedersen & Peters, 1971), therefore conditions must

have existed for growth of antral follicles; however, follicles starting their growth earlier terminated in atresia.

A striking feature of the data is the numbers of atretic follicles observed in the treatment group. The numbers of atretic follicles found at stages V, VIa and VIb were significantly greater in the progesterone treated group when compared with controls at all times. The percentage contribution of atresia to these three stages of follicles was consistently higher in the progesterone treated group. If data for the ages 60-100 days are combined, control animals have a larger percentage of atretic follicles at stages V and VIa than at VIb, thus indicating that selection procedures are occurring in these follicles. The data expressed in terms of treatment time showed a decrease in atresia at VIb in the treated groups.

Since there were no ovulations in the progesterone treated animals all stage V+ follicles must succumb to the atretic process. The increase in atresia in the treatment group is probably a result of low concentrations of gonadotrophins. In the cycling animal it has been estimated that a follicle takes 4 days to become atretic (Byskov, 1974b) but atretic follicles may persist for longer (Oakberg, 1979). The substantial increase in atresia observed at stage V+ in treatment groups could be as a result of follicles taking longer to degenerate and so these would persist longer and accumulate. There are indications from other studies that degenerating antral follicles may persist longer in pregnant and pseudo-pregnant rats (Osman, 1985; 1986). The decline in the number of healthy stage V+ follicles and the resultant increase in atretic follicles may be influencing events earlier in

follicular development perhaps by alleviating an inhibitory influence on the rate of growth of small follicles. The most likely level of control would be one operating at small intermediate growing stages as a direct influence on the non-growing primordial population is more difficult to explain. It would be advantageous to the animal to always have enough follicles capable of being selected for ovulation under suitable conditions.

Mechanisms by which the suppression of ovulation by increased levels of progesterone exert the observed effects on follicular dynamics are difficult to postulate since it would appear that these are occurring at different levels of control. There are three levels at which effects are probably being exerted at different times during treatment 1) The initiation of growth from the primordial pool, 2) Growth rate of small follicles and 3) deaths at larger stages of follicular development. Changes in some of these factors, but not necessarilly all of them, may be directly related to the increase in progesterone, either directly or by its action on gonadotrophin levels whilst others may be as a result of intraovarian factors due to the altered relationships in the follicle population. Intra ovarian and paracrine factors are likely to be important, however the mechanism of action of factors such as follicular regulatory proteins and interactions with these and steroid hormones are not fully understood. It is perhaps due to our lack of knowledge of small growing follicles and their interactions with hormonal factors that precludes the suggestion of any general mechanisms to explain the results obtained here.

In conclusion, this study has found effects at all levels of follicular development after suppression of ovulation by progesterone. It is unlikely that progesterone acts directly on the primordial population to increase the initiation of growth, but this increase may be related to the effects of the large stages, and it is possible that progesterone has a direct effect on the growth rate of small follicles. The increase in atresia may be as a result of decreased gonadotrophin levels inhibiting the follicle from undergoing final maturational changes. Differences may exist at primordial and stage V follicles between long and short term treated animals, however more information will be required to determine the character and underlying causes of these differences. Because of the complexity of the relationships involved in this situation, new methods will have to be devised to elucidate the mechanisms involved especially where the small growing follicles are concerned. The isolation and culture of specific follicle stages, in some cases by combining different stages, together with in vivo work, could be important ways forward in aiding our understanding of these complex interactions.

Chapter Five:
Clonal analysis of mouse ovarian follicles.

Introduction:

The aim of this study was to estimate the number of clones that give rise to the granulosa cell population in the ovarian follicle. To achieve this we have utilised a technique which has been used extensively by developmental biologists for determining the origins, lineages, and number of cells founding a particular tissue during development, to determine the size and clonality of the follicle progenitor cell population.

This technique employs genetic markers, of which many examples exist in laboratory mice (see West, 1984). Natural cell markers arise as a consequence of tissue mosaicism, with most of this being as a result of random inactivation of the X chromosome (Lyon, 1974). Early in development of the female embryo one of the two X chromosomes is inactivated as a gene dosage compensation mechanism. Thus the daughter cells express only the genes of the active X chromosome (Monk & Harper, 1979). This inactivation is apparently irreversible in somatic cells, but in the germ line reactivation occurs during oogenesis shortly before meiosis commences (Chapman *et al.*, 1982). However, new evidence now exists to suggest that X chromosome inactivation in somatic cells becomes less stable with ageing (Wareham *et al.*, 1987).

When an animal is heterozygous for the alleles of any particular X chromosome encoded gene, its tissues are composed of clones of cells expressing one or other allelic product. When relatively little cell mixing occurs the clones remain topographically localised in patches. The size and number of these patches depend on the number of cells founding the tissue and the number of divisions required for the final

differentiated state, assuming that cell death is negligible (Ansell & Micklem, 1986).

This study utilises the electrophoretic variant of the X linked enzyme phosphoglycerate kinase (PGK-1) which was discovered by Nielsen & Chapman (1977). An improved method of measuring PGK-1 activity was introduced in 1980 (Bücher *et al.*, 1980). Phosphoglycerate kinase is an enzyme of the glycolytic pathway, a variant of this enzyme which shows different electrophoretic motility was found in a wild mouse population in Denmark and designated PGK-1A (Nielsen & Chapman, 1977; West & Chapman, 1977) in contradistinction to the familiar form, PGK-1B. The respective alleles *pgk-1*^{a} and *pgk-1*^{b} now exist on genetic backgrounds of several inbred strains of mice.

Products of the PGK-1 alleles are good markers for the estimation of the clonal composition of tissues because they are expressed in all tissues and they are X-chromosome linked (Kozak *et al.*, 1974; Ansell & Micklem, 1986) and exist on a number of congenic backgrounds. This marker has been successfully used to study the theory that haematopoiesis in the mouse can be supported by a small number of clones (Burton, *et al.*, 1982; Micklem *et al.*, 1987).

The technique rests on the assumption that once the progenitors for a given tissue have been determined each clone multiplies and undergoes cell death at the same rate. The clones must therefore contribute equally to the resulting adult structure, and for any given structure there should be some minimum number of cells required.

The process by which the oocyte is enclosed by layers of somatic cells is known as folliculogenesis. A follicle is formed when the presumptive granulosa cells surround the oocyte and an intact basal lamina encloses the unit (Peters, 1978). It is not known whether the cells surrounding the primordial follicles are derived from a single clone or a number of clones, and, in the latter case, the number of clones involved. The former situation could arise from the association of a single progenitor cell with the oocyte followed by multiplication to produce the characteristic single layer. Alternatively a monoclonal follicle could arise from the association of the oocyte with a number of cells which share a common ancestral cell.

The granulosa cells multiply to form a solid follicle with several cell layers and subsequently undergoes a morphogenetic transition in which the extracellular space expands to form the Graafian follicle. The epithelium of the Graafian follicle is uniform in thickness except at the pole where granulosa cells support the oocyte. Thus, two morphologically distinct sub-populations exist: those forming an epithelial lining of the follicle (mural granulosa cells) and those in which the oocyte is embedded (cumulus granulosa cells). Differences in secretory products have been found in cumulus and mural granulosa cells. Mural granulosa have been found to secrete plasminogen activator but cumulus cells do not (Gilula et al., 1978). Distinct populations of granulosa cells have been identified by differences in antigenicity (Erickson *et al.*, 1985). Whether these sub-populations of granulosa cells have had distinct developmental lineages is unknown.

The developmental question of interest in the follicle is whether each of these individual units are derived from the association of a single cell with the oocyte and this cell being the precursor for all subsequent cells of the follicle. Such a situation would result in the follicle having a monoclonal origin. The other situation would be where a few cells around the oocyte divided to produce the surrounding follicle layers and this would be termed a polyclonal origin.

In the event that the granulosa population is polyclonal there is the possibility that only one clone becomes associated with the oocyte in the form of the cumulus oophorus. Apart from the basic importance of discovering cell lineages in developmental biology the question is of interest because metabolic coupling exists between the oocyte and granulosa cells (Moor, 1983). Overall granulosa and oocyte metabolism could in theory therefore differ in neighbouring follicles depending on which X chromosome was active in the granulosa cells. Using the technique of cell lineage markers we are able to estimate the number of clones in the granulosa cell population and in the cumulus mass of cells surrounding the oocyte.

Materials and Methods:

Isolation of granulosa cells:

Female CBA/Ca mice which were heterozygous at the PGK-1 locus and aged between 21-28 days old were obtained from Dr J.D. Ansell of the Department of Zoology, University of Edinburgh. They were given an injection of 10 iu of Pregnant Mares' Serum Gonadotrophin (PMSG) and 24 hours later antral follicles were isolated under a dissecting microscope

by dissection in Hepes buffered culture medium 199 (Flow Laboratories, Irvine). Individual follicles were removed and placed in microdrops of media which were covered with liquid paraffin. The follicles could then be manipulated and a maximal recovery of cells effected. To obtain granulosa cells in aggregate (i.e. cumulus cells and mural granulosa cells) the follicle wall was ruptured using a fine needle and the follicle cells expressed. These cells were drawn up with gentle suction into a finely drawn capillary tube and transferred to Eppendorf tubes (1.5ml). The oocyte and theca-stroma cells were discarded.

Isolation of Cumulus mass and mural granulosa cells:

The procedure for the removal of these cells was the same as that described above, except that the follicle was ruptured in such a way as to release the oocyte-cumulus mass intact. This mass was then pipetted into a fresh microdrop in which the oocyte was removed and discarded before pipetting the remaining cells into an Eppendorf tube. Using this method, there was little or no contamination with mural granulosa cells.

The cells were centrifuged at approximately 18000g for 40 seconds in an Eppendorf centrifuge. Excess fluid was removed by carefully blotting the side of the tube. The cells were resuspended in 7µl of sample buffer (50mM-triethanolamine hydrochloride (Sigma), 0.3mg/ml dithioerythritol (Sigma) 0.5 mg/ml bovine serum albumin; 2mg/ml digitonin (Sigma), and vortex mixed before being snap frozen in dry ice. All samples were stored at -70°C until they were assayed for PGK activity.

PGK ASSAY:

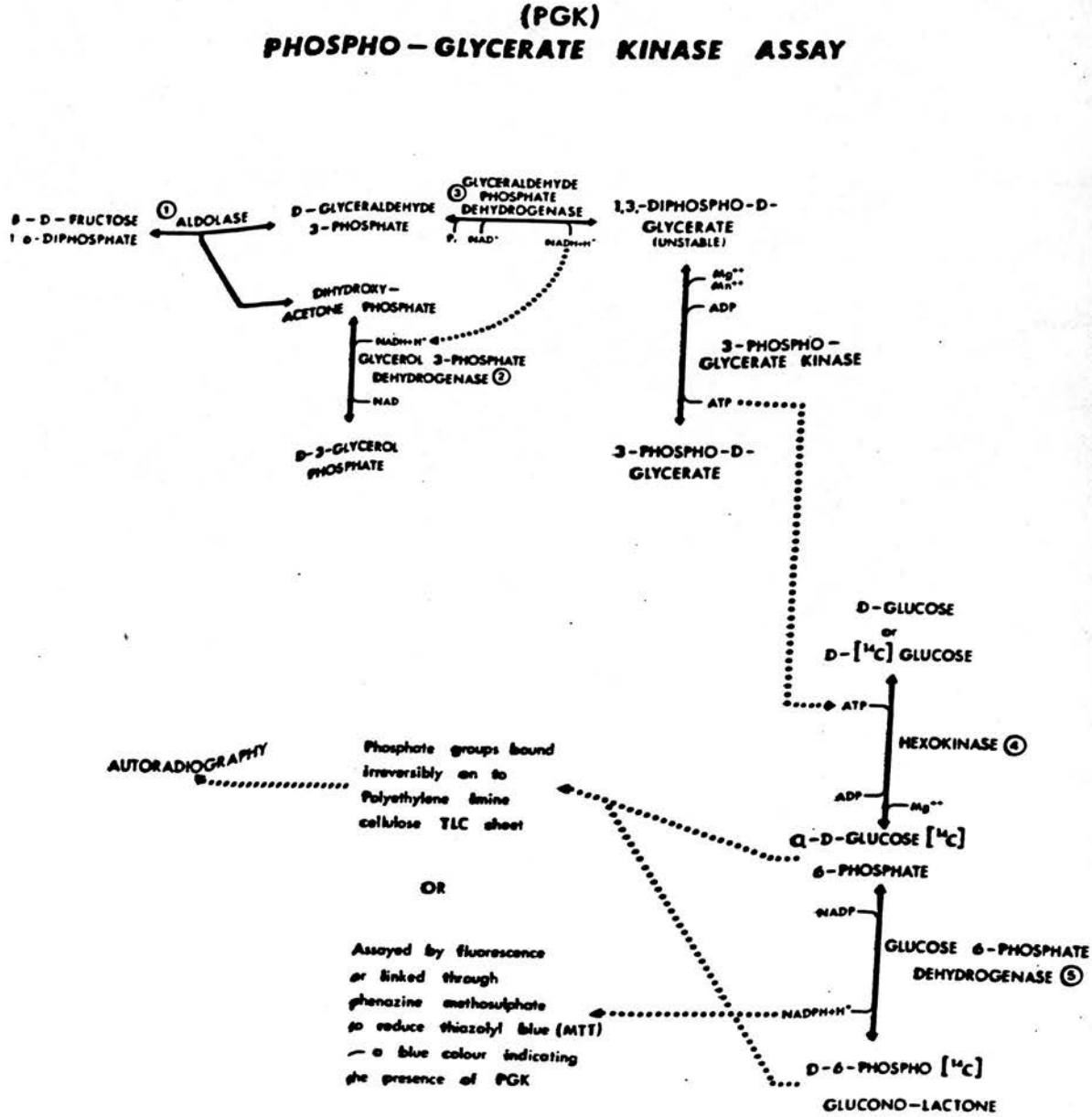
PGK-1 alloenzymes were separated by electrophoresis for 40 minutes at 250 volts at pH 8.8 on thin layer cellulose acetate membranes using a cooled tank and a bridge distance of 9cm. Immediately after electrophoresis the membranes were applied to a polyethylene imine (PEI) thin layer cellulose sheet on which had been spread a solution of enzymes, substrates and co-factors necessary for visualising PGK activity (Ansell & Micklem, 1986). ^{14}C labelled D-glucose was added as a tracer. In these experiments the substrate for PGK-1, 1,3.-diphospho-D-glycerate, is unstable and has to be generated *in situ* during incubation of the membranes. This is achieved by addition of fructose 1-6 diphosphate, NAD, ADP and enzymes I, II and III. The ATP generated by PGK-1 action is used in the phosphorylation of glucose (see figure 5.1). The radioactive final products of the linked enzyme reactions (glucose-6-phosphate + 6-phosphogluconolactone) were adsorbed onto the PEI sheet which were then autoradiographed on Kodak XS X-ray film (Ansell & Micklem, 1986). The autoradiographs were scanned with an automated microdensitometer and the area under each peak integrated in order to estimate the proportion of A and B alloenzyme in the lysate.

Repeatability:

It is important to obtain an indication of the repeatability of the method as results were obtained from different runs. In order to determine the degree of repeatability of the technique and the contribution of inter-assay variation to the total variance a single sample of mouse bone marrow was run several times on the electrophoresis system. This tissue was used because of the abundance of material (cf. follicle cells).

Figure 5.1:

Diagrammatic representation of various steps involved in the assaying of PGK alloenzymes (Ansell & Micklem, 1986).



Data analysis:

The values obtained for each sample of cells from individual follicles were expressed as a percentage value of the two alleles a and b. The mean and the variance for these percentage values were obtained and binomial statistics applied.

Binomial equation:

$$n=p(1-p)/\sigma^2$$

Where p = the mean value of the A allele

1-p = the mean value of the B allele

σ^2 = the sample variance

n = the mean number of clonal precursors

The estimate of variance between samples will indicate the number of clones of cells contributing to the tissue. If only one cell founds the tissue its enzymatic phenotype will either be A or B. If many cells seed the tissue the ratio of A-B will be consistently near to 50:50 in all samples. Intermediate situations can be analysed by application of binomial statistics to the variance of phenotypes between tissues. The higher the variance the lower the number of precursor cells.

Microdensitometry of nuclear DNA:

Since polyploidy has been reported in porcine granulosa cells (Coulson, 1979) it was necessary to determine whether this occurred in murine granulosa cells as this may interfere with the interpretation of the results. The DNA content of murine granulosa cells was tested by microdensitometry after staining by Feulgen's method (Drury &

Wallington, 1967). Suspensions of granulosa and liver cells as a control were fixed on separate clean slides using methanol:acetic acid (3:1,v:v) for 5 minutes. They were air dried, hydrolysed in 1N hydrochloric acid for 15-20 minutes and stained with aldehyde fuchsin. The preparations were scanned and values integrated using a Vickers micro densitometer set with an emission wavelength of 560nm and a 50 X objective lens.

Three dimensional reconstruction of primordial follicles:

The number of cells surrounding a primordial follicle was determined in serial histological sections. Camera lucida drawings were made in order to gain an impression of the follicle in three dimensions. The ovaries of ten 21 day old CBA/Ca mice were used. They were fixed in aqueous Bouins' fluid for 24 hours, dehydrated and embedded in epoxy resin. Sections were cut with a glass knife at 3μ in a series of at least ten. The sections were stained with haematoxylin. Plastic sections were obtained for this purpose to minimise tissue shrinkage and distortion artefacts. By reconstruction from drawings the number of granulosa cells surrounding the oocyte at this stage of follicular development was determined.

Results:

Reproducibility of PGK measurements:

Figure 5.2 shows the results of running a sample of mouse bone marrow several times on the electrophoresis system and is therefore an indicator of the repeatability of the technique. The variance producd by this can be regarded as the variation intrinsic to the system. The mean

for this sample when analysed 70 times was found to be 68 with a range of 63-73%. The variance of these repeat figures was 5.6.

Ploidy of cells:

Figure 5.3 gives the results of the microdensitometry readings on granulosa cells and liver cells . The mean density for diploid cells was 11.1 and the results show that 94% of the granulosa cells fall into this category with around 65% of liver cells showing this. The remaining 10% of granulosa cells show double this density of DNA and probably represent those cells during the S and G_2 phases of the cell cycle. The higher proportion of hepatic cells with greater than the expected 2c DNA level was expected since, although there are relatively few divisions in non-regenerating liver, tetraploid and even higher ploidies are present.

Granulosa cells:

75 follicles yielded samples which were satisfactory for running individually on the gel electrophoresis system. The results are shown in table 5.1. This table gives the percentage of cells from the sample showing the A and the B allele of the PGK-1 enzyme and the number of samples. Using the mean (X) and the variance (σ^2) of the percentage A and B we are able to apply the binomial equation and from this derive the number of precursor cells which would be required to obtain this mean percentage distribution of the alleles. A small proportion of follicles (12%) contained a monoclonal population of granulosa cells (either alloenzyme A or B).

Figure 5.2:

Reproducibility of assay. Each gel represents a single samples of bone marrow run several times and the percentage of PGK 1A estimated.

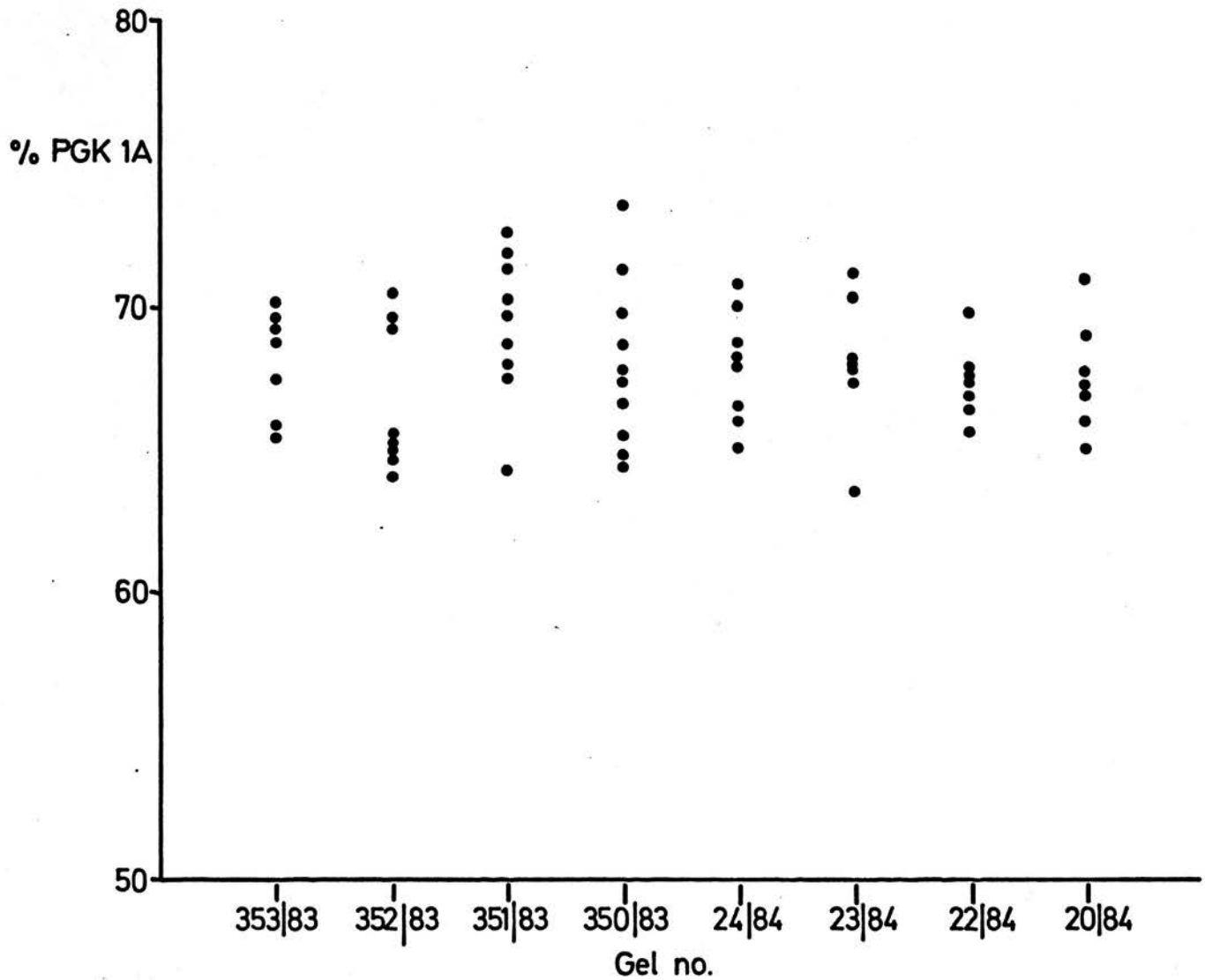


Figure 5.3:

Optical microdensitometer measurements of liver cell nuclei (controls) and granulosa cell nuclei. Density is in arbitrary units

The mean density for diploid cells = 11.1; 94% of granulosa cells in this sample fell into this category.

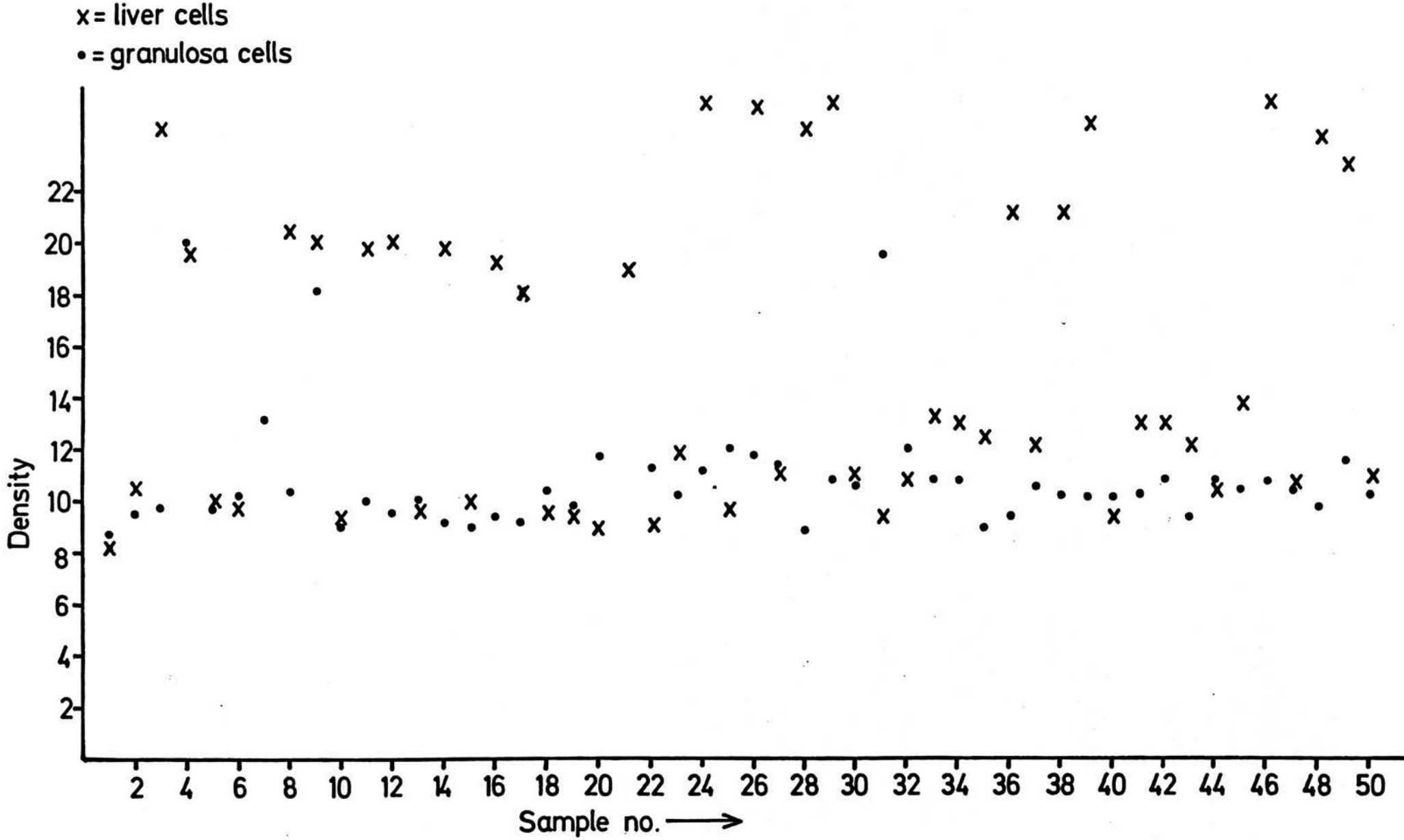

x = liver cells
• = granulosa cells
Density
2
4
6
8
10
12
14
16
18
20
22
2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32 34 36 38 40 42 44 46 48 50
Sample no. ⟶

Table 5.1:
Proportion of A and B alloenzymes found in samples of granulosa cells (cumulus + mural) from 75 follicles.

Granulosa cells		Number of samples
A	B	
100	00	7
91	9	2
90	10	2
89	11	1
86	14	1
85	15	3
83	17	2
82	18	2
81	19	2
80	20	2
79	21	3
78	22	2
77	23	2
76	24	2
75	25	2
74	26	2
73	27	2
72	28	1
71	29	3
70	30	1
68	32	2
67	33	2
66	34	1
65	35	2
63	37	1
62	38	1
61	39	2
60	40	1
57	43	1
55	45	3
51	49	1
50	50	1
47	53	1
46	54	2
43	57	1
41	59	1
39	61	2
37	63	2
33	67	1
27	73	1
00	100	2
X 69	31	N=75
σ^2 443		

Binomial equation:

$$n = \frac{p(1-p)}{\sigma^2} = \frac{69\ (31)}{443} = 5$$

Where p = mean of A. n= number of clonal precursors.

The results show that there is a dominance of the A allele with mean values of 69% A and 31% B allele. From the binomial equation (n=69(31)/443) it was estimated that the number of clonal precursors is approximately 5. Figure 5.4 shows the frequency distribution of the PGK-1A allele from these 75 samples and majority of the samples contained between 61-90% of the A allele.

Cumulus mass and Mural granulosa cells:

Whether or not the clonal descendents of the granulosa cell precursors are distributed randomly distributed between mural and cumulus sub-groups can be determined by examining the allozymal phenotypes. Table 5.2 shows the results from samples of cumulus cells and mural granulosa cells from 34 individual follicles. The results show the percentage of the A allele and of the B allele in each of these cell types. When these results were applied to the binomial equation (n=74.5(25.5)/367) a mean value of 5.17 was obtained for the clonal size of the precursor population of mural granulosa cells. This value was not significantly different from that obtained from the aggregated granulosa cells in the previous experiment. When the same analysis was applied to the results from the cumulus mass of cells (n=77(23)/578) then a value of 3 is obtained for the precursor population size.

The proportions of total enzyme activity which was due to PGK-A was compared for the mural and cumulus cell sub-groups using the method of linear regression analysis (Fig.5.5). The proportions were highly correlated (r=0.899) indicating that both cell types are derived from the same precursor pool.

Figure 5.4:

Frequency distribution of the percentage of phospho-glycerate kinase activity (PGK) which is of the A phenotype in granulosa cells taken from 68 individual follicles. This distribution is comparable with that for other tissues, with a mean greater than 50% being expected because of the presence of alleles of an X inactivating locus (Xce). This affects the probability that a particular X chromosome will be inactivated (Cattanach & Papworth, 1981).

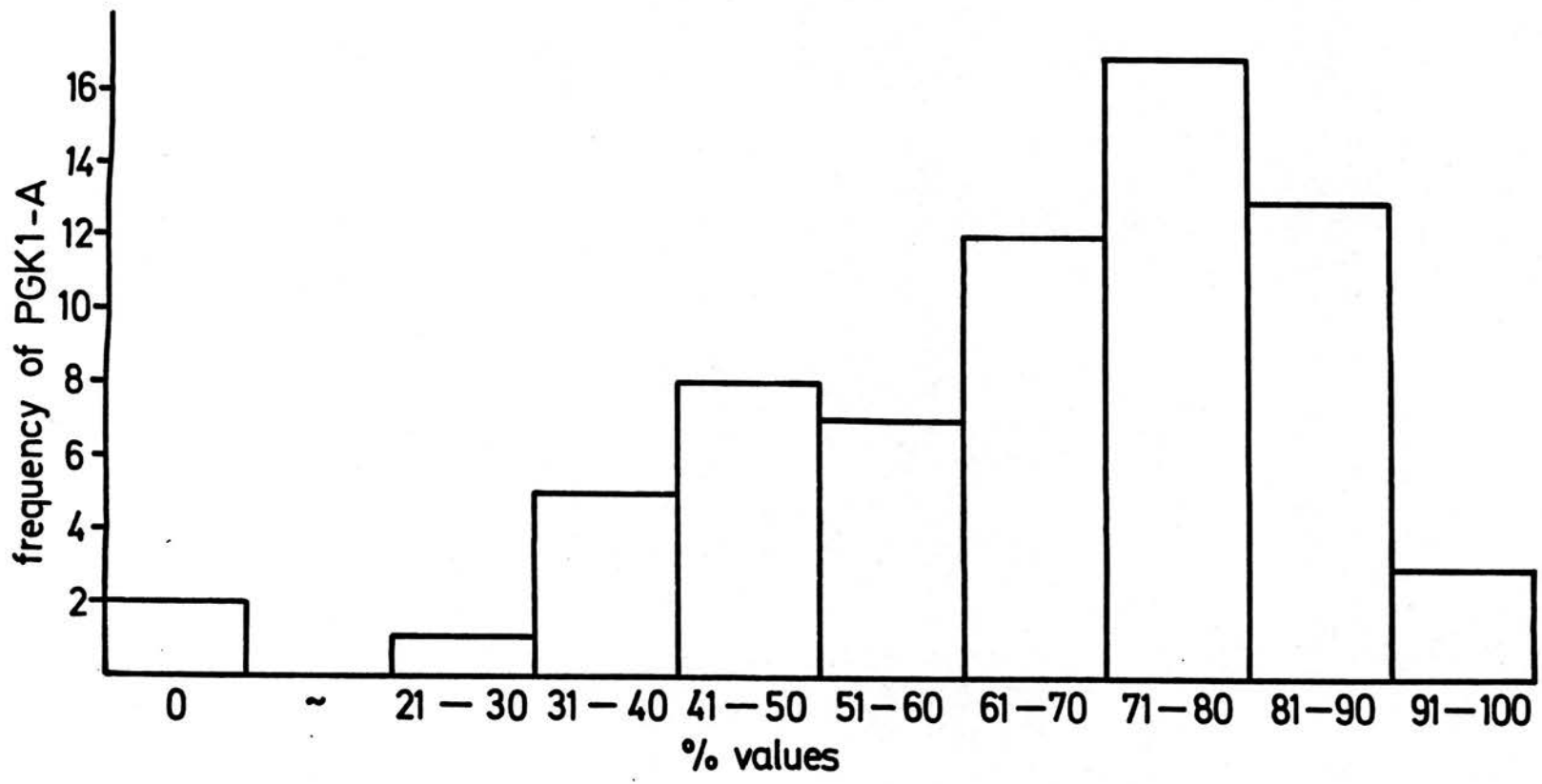

frequency of PGK1-A
16
14
12
10
8
6
4
2
0
~
21 — 30
31 — 40
41 — 50
51 — 60
61 — 70
71 — 80
81 — 90
91 — 100
% values

Table 5.2:
Proportion of A and B PGK alloenzymes found in samples of mural granulosa and cumulus cells from 31 follicles.

Granulosa cells		Cumulus cells		Number of samples
A	B	A	B	
100	00	100	00	2
100	00	-	-	1
97	03	100	00	1
95	05	87	13	1
95	05	80	20	1
94	06	100	00	1
93	07	88	12	1
91	09	100	00	1
89	11	91	09	1
86	14	100	00	1
86	14	83	17	1
84	16	100	00	1
82	18	86	14	1
80	20	76	24	1
77	23	71	29	1
72	28	-	-	1
70	30	87	13	1
68	32	62	38	1
68	32	74	26	1
66	34	-	-	2
65	35	74	26	1
64	36	-	-	1
61	39	40	60	1
58	42	-	-	1
53	47	46	54	1
42	58	-	-	2
41	59	21	79	1
24	76	23	77	1
$\bar{X}$ 74.5	25.5	77	33	
σ^2 367		578		

Binomial equation:

$$n = \frac{p(1-p)}{\sigma^2}$$

mural cells $= \frac{74.5\ (25.5)}{367} = 5.17$

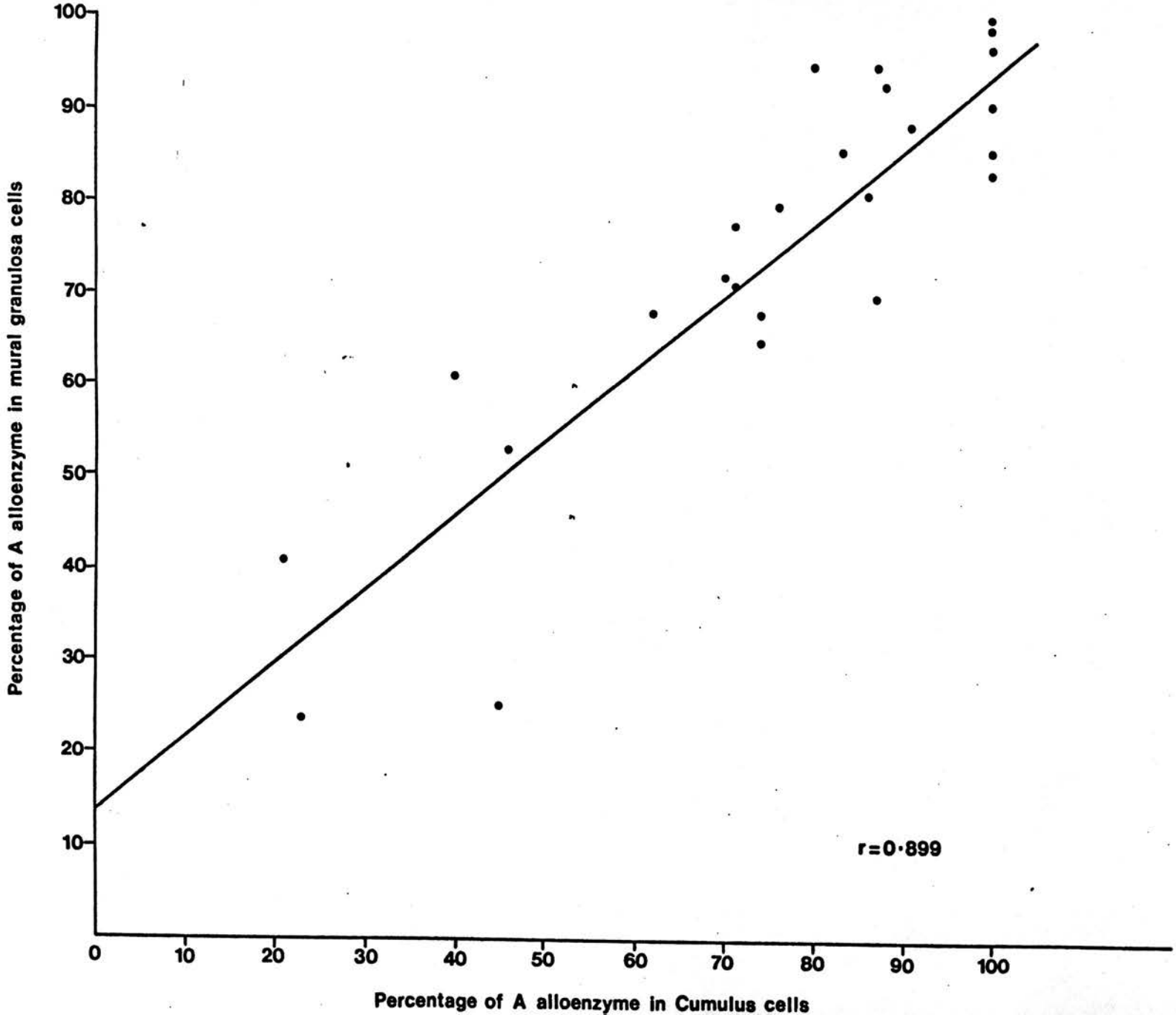
cumulus cells $= \frac{77\ (33)}{578} = 3$

(-) indicates no activity detected, probably as a result of too few cells being recovered.

Figure 5.5:

Correlation between the percentage of A alloenzyme found in cumulus and granulosa cells.

The regression line has been drawn by the method of least squares and the product-moment correlation coefficient (r) calculated. ($r=0.899$, $p<0.001$).


Percentage of A alloenzyme in mural granulosa cells
100
90
80
70
60
50
40
30
20
10
0
10
20
30
40
50
60
70
80
90
100
Percentage of A alloenzyme in Cumulus cells
r=0·899

When the cumulus mass and mural granulosa cells were treated separately 9% of the samples of granulosa cells appeared monoclonal and all were represented by the phenotype A. 20% of the samples of cumulus mass were of monoclonal origin (Phenotype A only) but these were not always the same follicles as those presenting a single phenotype in the mural cells.

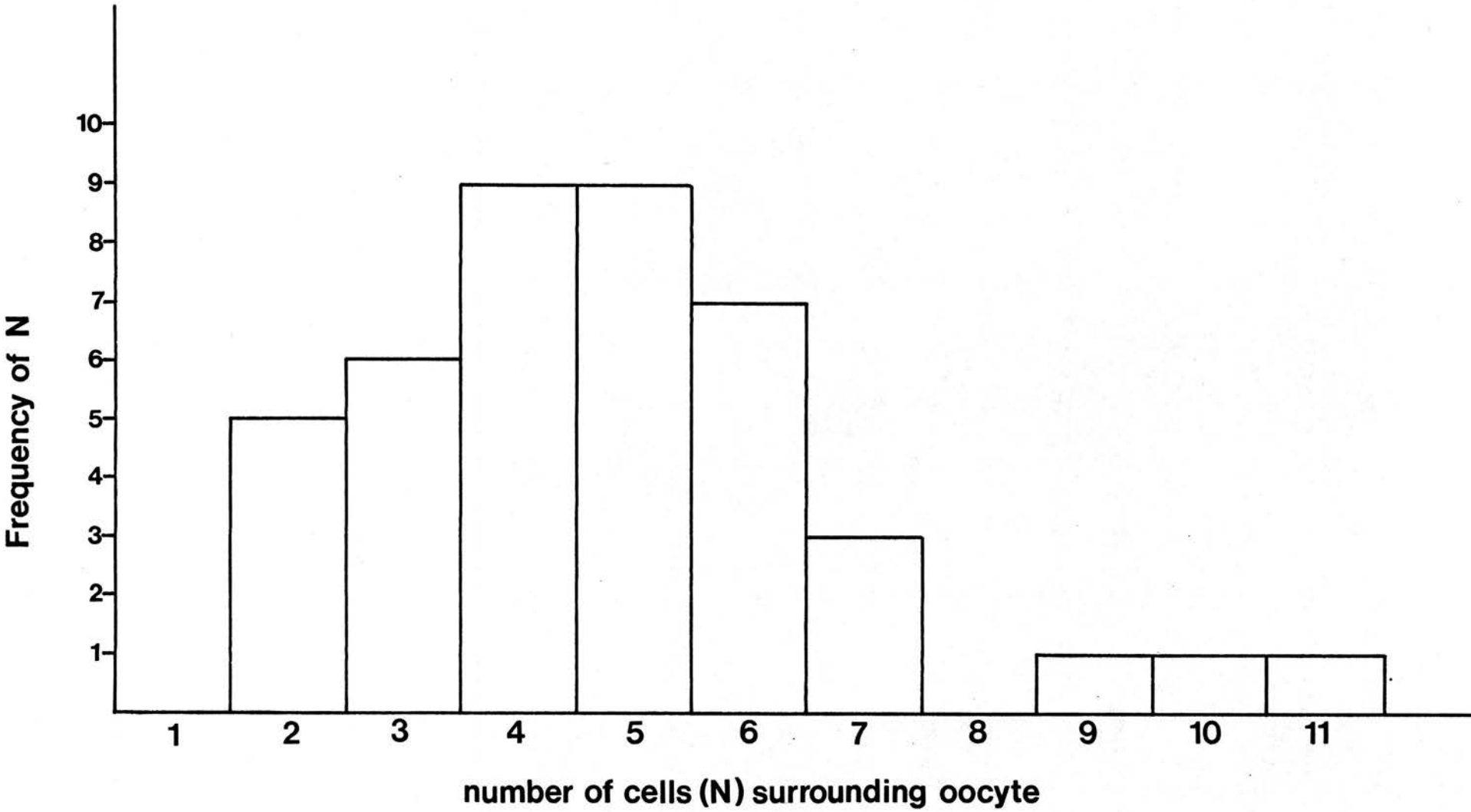
3-Dimensional reconstruction of follicles:

Figure 5.6 shows the results obtained through serially reconstructing 42 primordial follicles in order to count the number of presumptive granulosa cell precursors surrounding the oocyte. The frequency distribution shows that the majority of follicles comprised of 3-6 somatic cells surrounding the oocyte. The mean of these data was 4.8 with a standard deviation of 2.05 and a standard error of the mean of 0.3. This result corresponds closely with that obtained from the clonal studies. Occasionally "naked" oocytes were encountered.

Figure 5.6:

Frequency distribution of number of presumptive granulosa cells surrounding primordial follicles from 42 follicles.

Data was obtained from three dimensional reconstruction of primordial follicles using camera lucida drawings.


Frequency of N
10
9
8
7
6
5
4
3
2
1
1
2
3
4
5
6
7
8
9
10
11
number of cells (N) surrounding oocyte

Discussion:

This is the first study which has attempted to determine the founding granulosa cell number for individual ovarian follicles. Ovarian follicle present a unique situation in which the follicular epithelium is distributed in discrete units and in intimate association with the oocyte thus clonal analysis within and between individual units can be carried out.

A clone may be defined in the general sense as a single progenitor cell that gives rise to a group of cells or a group of cells all derived from a single ancestor. In this study clone is being used in the sense of the single cell that may give rise to defined populations of cells in the adult structure.

The results of this study indicate that a small number (about 5) of progenitor cells give rise to the granulosa cell population, which numbers about 50000 cells in the mature follicle. This estimation of number of cells founding the ovarian follicle is based on the application of the binomial statistic to the measured estimates of the frequency of the products of the two alleles. An individual cell will normally either express type A or type B phenotype. With each mitotic division the resultant daughter cells will be of the same clonal origin and it is supposed that this process is binomial (Stone, 1984) in that each division (also referred to as a marking event) the resulting clone is independently type A or B, with constant probabilities p and q=1-p.

In this application the estimate of the variance between experimental samples is a good indication of the number of cells contributing to the tissue. If only one cell founds the tissue its enzymatic phenotype (because of X chromosome inactivation) will either be A or B if a small number of clones are present the variance about the mean is expected to be greater than if a large number of clones are present. If many cells found the tissue the ratio of A-B will be consistently near to 50-50 in all samples. In this study the proportion of A allele was greater than 50% and this is to be expected because of the indirect influence of an inactivation centre present on the X chromosome (known as the X chromosome controlling element, Xce). Three alleles of Xce have been described and they affect the probability that a particular X chromosome will be inactivated. (Cattanach, 1975; Cattanach & Papworth, 1981).

In this study the majority of follicles had a polyclonal origin because both phenotypes of PGK were expressed within the granulosa cell population. A minority of follicles were mono-clonal in origin and it is possible that they could have originated from a single cell. It is also possible that these monoclonal follicles were formed by a few cells all of the same type, however this would be less likely if X chromosome inactivation was random as the probability that all of the cells contributing to a tissue were of the same type would be low.

The process of folliculogenesis occurs around the time of birth in the mouse (Peters, 1978), although some differences in this timing exists between strains. The origin of follicle cells has been a question for scientific study and debate for a long time. Kölliker (1898) ascribed to

the rete ovarii the role of contributing to follicle formation as the cells of the follicular envelope appeared to be continuous with those of the ovarian cord but this was dismissed by Winiwarter & Sainmont, (1908) who concluded from studies on the cat that only medullary follicles were invested with cord cells and these ultimately degenerated. More recently Byskov & Rasmussen (1973) noted the continuity between rete cells and cells of the follicular envelope and studies on the development of the rete ovarii in relation to follicle development in the neonatal mouse supported Kölliker's original assumption (Byskov & Lintern-Moore, 1973).

The trigger for the initiation of follicle growth is still unknown but early signs of growth are a change in shape of follicle cells from squamous to cuboidal with increase in cell number. At the onset of growth of the follicle there is an increase in RNA synthesis in the oocyte (Lintern-Moore & Moore, 1978). Subsequent growth involves multiplication of the granulosa cells to form a multilayered structure, which the present study shows represents a small number of clones. A major assumption of this study is that clones do not have differential rates of cell multiplication or death. In preantral follicle there are few pycnotic cells and from evidence of tritiated thymidine autoradiography, cell divisions are random (Pedersen, 1969; 1970a Gosden *et al.*, 1983) at least until the antrum has formed. In immature rats there is little differential multiplication even at Graafian stages (Hirshfield, 1986a).

The three-dimensional reconstruction of primordial follicles corroborate results of the PGK study, there being approximately 5 pre-granulosa

cells identified in both studies. This raises the possibility that the layer of pregranulosa cells observed around the oocyte are the same as those inferred by the PGK analysis and are thus the true precursors of the definitive granulosa cell population. Whether the monoclonal follicles only had a single cell associated with the oocyte cannot, however, be determined at this time. To approach this question it will be necessary to visualise the clones. It is hoped that this will be carried out in the near future with chimaeric tissues using cDNA probes. Such methods will also permit analysis of cell mixing at later stages in morphogenesis.

The morphological studies show that follicles with only one cell were rare, but this approach is subjective as the close proximity of a cell to the oocyte does not neccessarily imply a functional relationship between the two. There are also sampling problems, because although follicles were selected at random some were more difficult to follow through serially than others and these were abandoned. This morphological study was carried out to support the cell lineage study to find some confirmation of this approach to estimating progenitor cell number. This study does not imply that there is a strict number of progenitor cells required for follicle formation but that a small number of cells are involved in this process and these studies estimate that this number is around 5. The morphological study shows that follicles with less than 2 or greater than 7 surrounding cells rarely occur.

As the follicle enlarges by proliferation of the granulosa cells a fluid filled antrum begins to form. The fluid filled follicle at this stage

has a uniform wall of cells (mural granulosa cells) except at the point of attachment of the oocyte. The oocyte is surrounded by a cluster of cells (Cumulus). The separation of the follicle into these two functional components in this study was carried out to determine the clonal origin and number of cells forming the cumulus mass it also verified the results obtained from the earlier study. The results showed that the estimated number of clones in the cumulus cell sub-population corresponded closely with the number of unsorted granulosa cells. It is concluded from this that there must have been a certain amount of mixing of clones during pre-antral development and that more than a single cell gives rise to the cumulus. Few samples of cumulus cells were found to have a monoclonal origin.

This study rests on the assumption that granulosa cells are diploid, however, the ploidy of granulosa cells has been questioned (Coulson, 1979). Autosomal cells normally have a diploid complement of DNA (=2N) and cells in division may contain up to 4N while in the S and G_2 position of the cell cycle. Coulson claims to demonstrate that polyploidy exists in porcine and bovine ovarian granulosa cells and that it occurs with the highest values in granulosa cells from small follicles of immature ovaries. Coulson also observed a decrease to 2N values in Graafian follicles just prior to follicular rupture and concludes that these ploidy changes in granulosa cells may be an important part of the differentiation process. In this study using the technique of microdensitometry, as opposed to biochemical measurements of cell aggregates in Coulson's study, no evidence for polyploidy in murine ovarian follicles could be found. Thus it was assumed that these

cells contained the diploid complement of DNA apart from a small proportion which were probably undergoing cell replication.

From this study it is concluded that a small number of progenitor cells in the primordial follicle leads to the epithelial envelope of the Graafian follicle. The granulosa cell lining of the mature Graafian follicle is a heterogeneous structure in respect of morphology, distribution of hormone receptors and sub-populations of granulosa cells have been identified in the pre-ovulatory human follicle (Hartshorne, 1987). Differences in biosynthetic activity have been shown to exist across the follicular epithelium according to the distribution of several enzymes (Zoller & Weisz, 1978; Zoller & Enelow, 1983). How this small number of progenitor cells leads to this structure is still unknown. Whether these clones form functionally distinct groups of cells in the large follicle might be studied using chimaeric tissue to follow through clones of cells. Further studies using cell lineage markers to follow groups of cells through development may help identify differential growth, cell migration and the distribution of clonal descendants at major transition points in follicles, such as time of antral formation. The results of this study indicate that the existence of sub-populations of granulosa cells in the mature follicle cannot be explained entirely by cell lineages. Other factors such as cell position and interactions between the granulosa cells with the stroma-theca and oocyte should also be investigated.

Chapter Six:

Scaling of ovarian follicle sizes and follicle numbers.

Introduction:

To gain an understanding of the nature of a particular biological system individual organs and associated structures within a particular species are normally studied. Ultimately the aim of such studies is to determine how the information obtained from them fits into a pattern with information from other species. A statistical or graphical tool used for comparing animals quantitatively and viewing these patterns is allometry or the biology of scaling. Allometry has been defined in general terms by Gould (1966) as the study of size and its consequences. It is a descriptive device for describing changes in proportion whether morphological or chemical with increase in size. Simple allometry being when the formula, $y = ax^b$ holds for two variables y and x where x is often the body weight. The variables a and b are the proportionality constant and the allometric exponent respectively. The equation is most often used in its log form:

$$\log y = \log a + b \log x$$

Thus, if the logs of the two dimensions x and y obeying the law are plotted against one another the points lie along a straight line. In problems of scaling, in which structures or functions are related to animal size, body size is normally considered to be the independent variable (x). Let M represent mass in the allometric equation

$$y = aM^b.$$

In this equation the exponent b is called the body mass exponent. This is the slope of the straight line that represents the allometric equation in a plot on logarithmic coordinates. The proportionality

coefficient, a, is identical with the intercept of the regression line at unity body mass, or M=1 since 1 raised to any power remains 1 , thus giving y=a.

Scaling of the reproductive organs has received little attention, probably because of the lack of data from a sufficient range of species, although a number of variables associated with reproductive physiology such as litter size, age at puberty etc have been well documented (May & Rubenstein, 1984). Parkes, (1932) suggested that the size of Graafian follicles are related to body size. However, his conclusions were based on only seven species. In primates, testicular weights, which are indicative of spermatogenic capacity, present allometric variation, but superimposed upon this are the effects of sperm competition in some species (Harcourt *et al.*, 1981). One of the aims of this study is to build up a data base in order to test whether allometric relationships exist for a number of ovarian parameters and, hence, to test a number of hypotheses of physiological significance, e.g. such as whether follicular surface area is related isometrically to body mass and, thus, to the volume for hormone distribution.

Since reproductive strategies vary between species it would be interesting to compare the numbers and the rate of utilisation of follicles in a range of species. Ovarian follicles are a non-renewable resource and the numbers present at the onset of reproductive life must last for the duration of the lifespan. In humans ovarian failure occurs at menopause in mid-life (Gosden, 1985b), which could indicate that there is a relatively poor endowment of follicles at the onset of fertile life. Thus, the relationship between the follicular endowment of

a species and body size would be interesting to examine particularly in connection with ageing. This study has been designed to examine the relationship between the numbers of follicles and body weight and also with that of maximum lifespan. Questions that are raised in relation to longevity are whether the numbers of primordial follicles at the onset of adult life follow an allometric relationship, or are there differences imposed by the variation in reproductive strategies? In order to exclude variability in degree of growth and development only follicular populations of peripubertal ovaries were examined in this study since only follicles which are capable of contributing to fecundity will be present.

Materials and Methods:

This chapter is divided into two parts, part one dealing with scaling of follicular sizes and part two dealing with the scaling of numbers of follicles in various species in relation to body weight and longevity. Table 6.1 refers to the codes by which species are designated throughout the study, 22 of the species are used in part 1 but only 19 in part 2.

Source of Materials:

Data were obtained from young, adult nulliparous individuals from 23 species. Ovaries were obtained at autopsy or at ovariectomy and were fixed, embedded in paraffin wax, serially sectioned at 7 or 8μm and stained with haematoxylin and eosin.

Table 6.1: List of species investigated and code numbers used in Figures.

Code no	Common name	Specific name.	Order
1	Bandicoot	*Isoodon macrourus*	Marsupialia
2	Common shrew	*Sorex araneus*	Insectivora
3	Hedgehog	*Erinaceus europaeus*	"
4	Elephant shrew	*Elephantulus elephantulus*	"
5	Pipistrelle bat	*Pipestrellus pipistrellus*	"
6	House mouse	*Mus musculus*	Rodentia
7	Wood mouse	*Apodemus sylvaticus*	"
8	*Bank vole*	*Clethrionomys glareolus*	"
9	Field vole	*Microstus agrestis*	"
10	Norway rat	Rattus norvegicus	"
11	Guinea pig	Cavia porcellus	"
12	Plains viscacha	*Lagostomus maximus*	"
13	European rabbit	*Oryctolagus cuniculus*	Lagomorpha
14	Domestic cat	*Felis catus*	Carnivora
15	Domestic dog	*Canis familiaris*	"
16	Sheep	*Ovis aries*	Artiodactyla
17	Swine	*Sus scrofa*	"
18	Cattle	*Bos taurus*	"
19	Horse	*Equus caballus*	
		Perissodactyla	
20	Common marmoset	*Callithrix jacchus*	Primates
21	Rhesus monkey	*Macaca mulatta*	"
22	Human	*Homo sapiens*	"
23	Greater horseshoe bat	*Rhinolophus ferrumequinum*	Chiroptera

Measurements of ovarian follicles and oocytes:

The mean diameters of non-atretic follicles and of oocytes in Graafian follicles were obtained in the largest cross-section of the structure being measured. Maximum diameter and the diameter at right angles was measured and the average of the two used. Between 8-30 of these measurements were obtained for at least 4-8 individuals of each species. Three developmental stages of follicles were measured:

(1) primordial follicles in which a small oocyte was surrounded by a unilaminar squamous epithelium,

(2) solid, multilaminar follicles in which formation of pools of follicular fluid was incipient,

(3) mature Graafian follicles shortly before ovulation.

Among species exceeding 1 Kg in body weight, the larger Graafian follicle dimensions were obtained by measuring fresh material with calipers. The surface areas and volumes of Graafian follicles have been estimated because these variables could be physiologically more significant than the follicular diameter (D).

Surface area and volume of follicles:

Surface area and volume estimates were based on the assumption that follicles are spherical with surface areas corresponding to, $4\pi(D/2)^2$ and volumes corresponding to $1.33\pi(D/2)^3$. The morphological appearance of follicles supported this assumption in all cases except the domestic bitch in which the surface area presented for diffusion to and from the vascular theca will have been underestimated because of folding of the follicular wall.

Total Graafian surface area and volume were calculated by multiplying the values for an individual follicle by the mean ovulatory quota for each species.

Body Weights:

The body weights for young adult individuals were obtained during the course of the study or were obtained from published sources as were ovulation rates (Altman & Dittmer, 1972).

Section two: follicle counts:

Data on follicle numbers were obtained from non-pregnant representatives of 19 species among eight orders. Depending on the size of the organ, every 10th, 20th or 40th section was examined and the number of primordial follicles counted. The nucleolus, if single and distinct, was used as a marker, otherwise the nucleus was used. Total numbers of follicles were estimated by multiplying the follicle counts by both the sampling frequency and a correction factor (Abercrombie, 1946).

Growing follicles from unilaminar to Graafian types were counted in the same sections. Atretic follicles were included in the counts when the marker remained distinct. Because of problems in obtaining suitable material data from the following 5 species were extracted from published studies in which comparable cytological methods had been used. Those for domestic swine and cattle were obtained from Erickson (1966; 1967). In laboratory mice (strain A) and rats and humans, the numbers of follicles at pubertal ages were obtained from regression lines, which were based on large sets of data which

extended across most of the lifespan (Block, 1952; Mandl & Shelton, 1959; Jones & Krohn, 1961a). The regressions were obtained using an exponential model, $N = Ae^{-bt}$ which produced a good fit with each data set and enabled the estimation of follicle numbers at puberty and the fractional disappearance rates for small follicles in adult life.

Adult lifespan:

The number of follicles present at onset of adult life have been examined in relation to maximum longevity of species after puberty. In most species, there was sufficient reliable data for estimating the maximum adult lifespan by subtracting the characteristic pubertal age from maximum longevity.

Analysis of Data:

The mean values for each of the variables were used for analysing interspecific patterns. They were transformed to common logarithms for graphical representation and for testing whether relationships were consistent with the allometric formula, $y=aM^b$. Linear regression analysis was carried out by the methods of least squares and the 95% confidence limits were calculated (Sokal & Rohlf, 1981). Product-moment correlation coefficients (r) were calculated to demonstrate possible relationships between each of the measured variables. The coefficient of determination (r^2) was also calculated; this being a useful measurement when the relative importance of correlations of different magnitudes are being considered (Sokal & Rohlf, 1981). The results were analysed using the statistical package "Microstat" (Ecosoft, Inc. Indianapolis).

Results:

Table 6.2 shows the mean follicular dimensions obtained for each of the species observed. The values for body weight and mean ovulation number used in this study are also given for the 23 species. Missing data are due to the unavailability of suitable material. These parameters are illustrated graphically and the relationships will be dealt with in turn.

Correlation coefficients were obtained for the major variables and these are presented in table 6.3. Statistically significant correlations, at the 5% level of significance, are shown in brackets. Highly statistically significant correlations occurred between body weight and number of primordial follicles, Graafian follicle diameter, individual and total Graafian follicle volumes and surface areas. and also the number of primordial follicles and the diameter, volume and surface area of Graafian follicles.

Primordial follicle diameter: body weight:

Diameters of primordial follicles ranged from 0.014-0.093mm and are statistically significantly correlated with body size and with both the numbers present at young adult ages and with the sizes of Graafian follicles and of oocytes (table 6.3). When plotted against body weight on logarithmic coordinates a linear correlation was obtained ($p<0.01$) in which the coefficient of determination (r^2) was 0.45 (Fig 6.1).

Table 6.2:
Mean values of follicle dimensions in microns and body weights in kilograms. List of species codes is given in Table 6.1

Species	primordial diameter	oocyte diameter	Graafian diameter	Body weight	ovulation number
1	32.0	133	844	.850	3
2	16.5	71	315	.006	8
3	25.0	59	800	.680	5
4	-	35	350	.069	98
5	17.0	68	-	.005	1
6	14.0	68	600	.020	8
7	17.0	59	500	18.000	6
8	17.5	60	455	.020	4
9	17.0	68	509	.022	4
10	16.0	63	900	.200	10
11	22.0	58	946	.700	4
12	27.0	60	276	3.000	300
13	85.0	93	930	4.000	8
14	93.0	112	1091	3.000	4
15	26.0	78	4500	10.000	4
16	37.0	113	6500	51.500	1
17	34.0	98	7500	250.000	10
18	46.0	77	20000	500.000	1
19	42.0	94	50000	408.500	1
20	35.0	78	4000	.350	2
21	39.0	104	6300	3.200	1
22	29.0	93	22000	60.000	1
23	-	55	350	.025	1

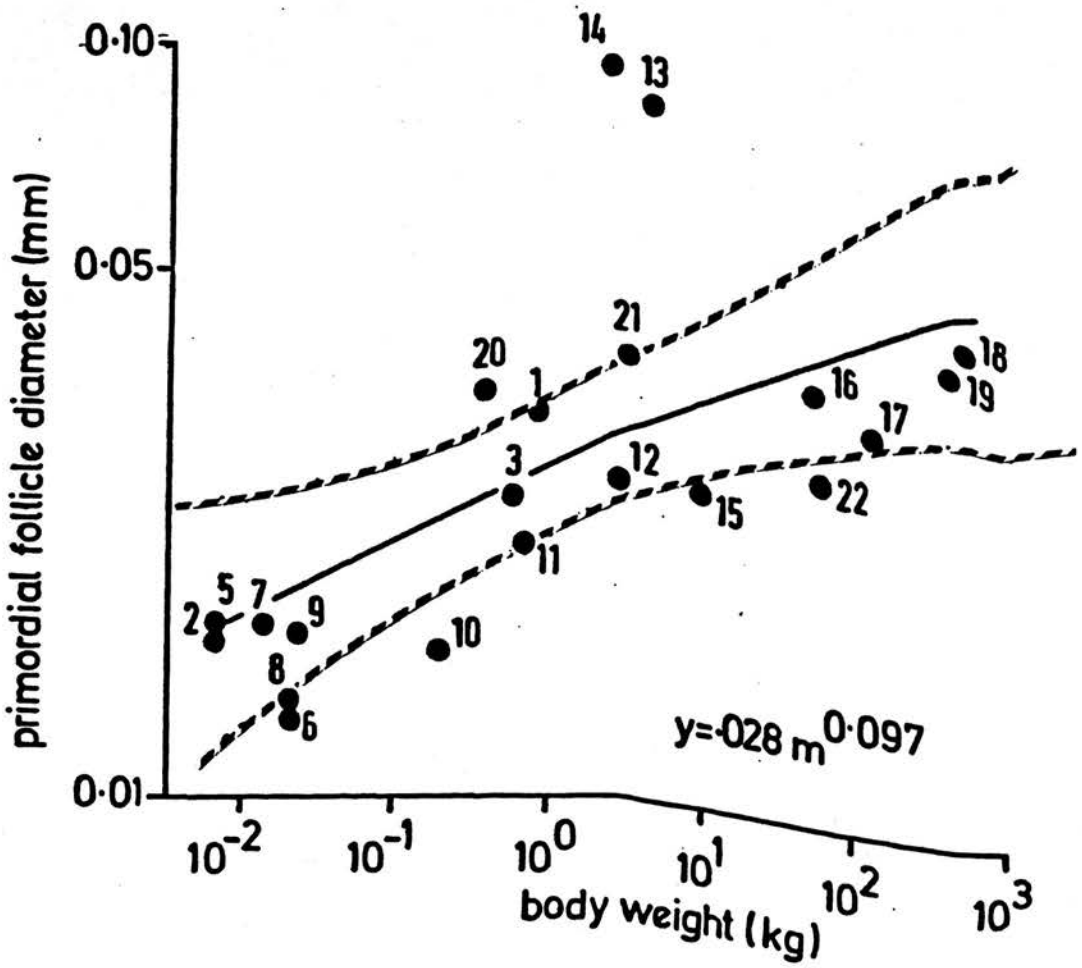
Table 6.3:
Correlation matrix for logarithmically transformed values for body weight and ovarian parameters.

					Diameter			Surface area		Volume
		Body wt.	Number of primordial follicles	Ovulation rate (n)	ovum	primordial follicle	Graafian follicle(GF)	GF*l	GF*n	GF*l
Number of primordial follicles		(0,953)								
ovulation rate (n)		-,270	-,462							
Diameter	ovum	0,552	(0,730)	-,422						
	primordial follicle	(0,669)	(0,676)	-,308	(0,769)					
	Graafian follicle (GF)	(0,880)	(0,902)	(-,673)	(0,687)	0,575				
Surface area	GF*l	(0,868)	(0,905)	(-,684)	(0,691)	0,561	(0,990)			
	GF*n	(0,921)	(0,930)	-,475	(0,686)	0,569	(0,958)	(0,967)		
Volume	GF*l	(0,869)	(0,905)	(-,684)	(0,691)	0,561	(0,990)	(1,000)	(0,967)	
	GF*n	(0,921)	(0,928)	-,558	(0,694)	0,571	(0,978)	(0,987)	(0,995)	(0,987)

Figure 6.1:

Variation between mean diameters of primordial follicles and body weight. The regression line with 95% confidence limits is presented along with the allometric formula.

Table 6.1 gives species code list for figures.



The interspecific differences in sizes of these follicles were mainly due to the volumes of ooplasm, which were disproportionately large in relation to body size in rabbits and cats.

Oocyte diameter in Graafian follicles:body weight:

Figure 6.2 shows the relationship between body weight and ovarian oocyte diameter in uniovular Graafian follicles. These were correlated at the 5% level of statistical significance ($0.05>p>0.02$). The diameter of oocytes in Graafian follicles varied among eutherian mammals from 0.058mm in small rodents to 0.113mm in sheep; in marsupial species it was 0.133mm. A regression line has not been fitted to this data since a linear relationship does not seem appropriate.

Size of follicle at onset of antrum formation : body weight:

Figure 6.3 shows the plot of log body weight against the diameter of follicles at the onset of extracellular fluid accumulation in 16 species. In spite of the range of body weights the antrum was incipient in follicles of 0.2-0.4mm diameter in all cases. Measurements of granulosa cells indicated that granulosa cell volumes were similar in all species, thus, the transition to antral stage must occur when similar number of cells have accumulated.

Graafian follicle surface area : body weight:

Individual Graafian follicle surface area and total Graafian follicle surface area were correlated significantly with body weight, giving correlation coefficients of 0.868 and 0.921 respectively.

Figure 6.2:

Differences between mean diameters of oocytes in Graafian follicles and body weight.

A regression line was not appropriate for these data.

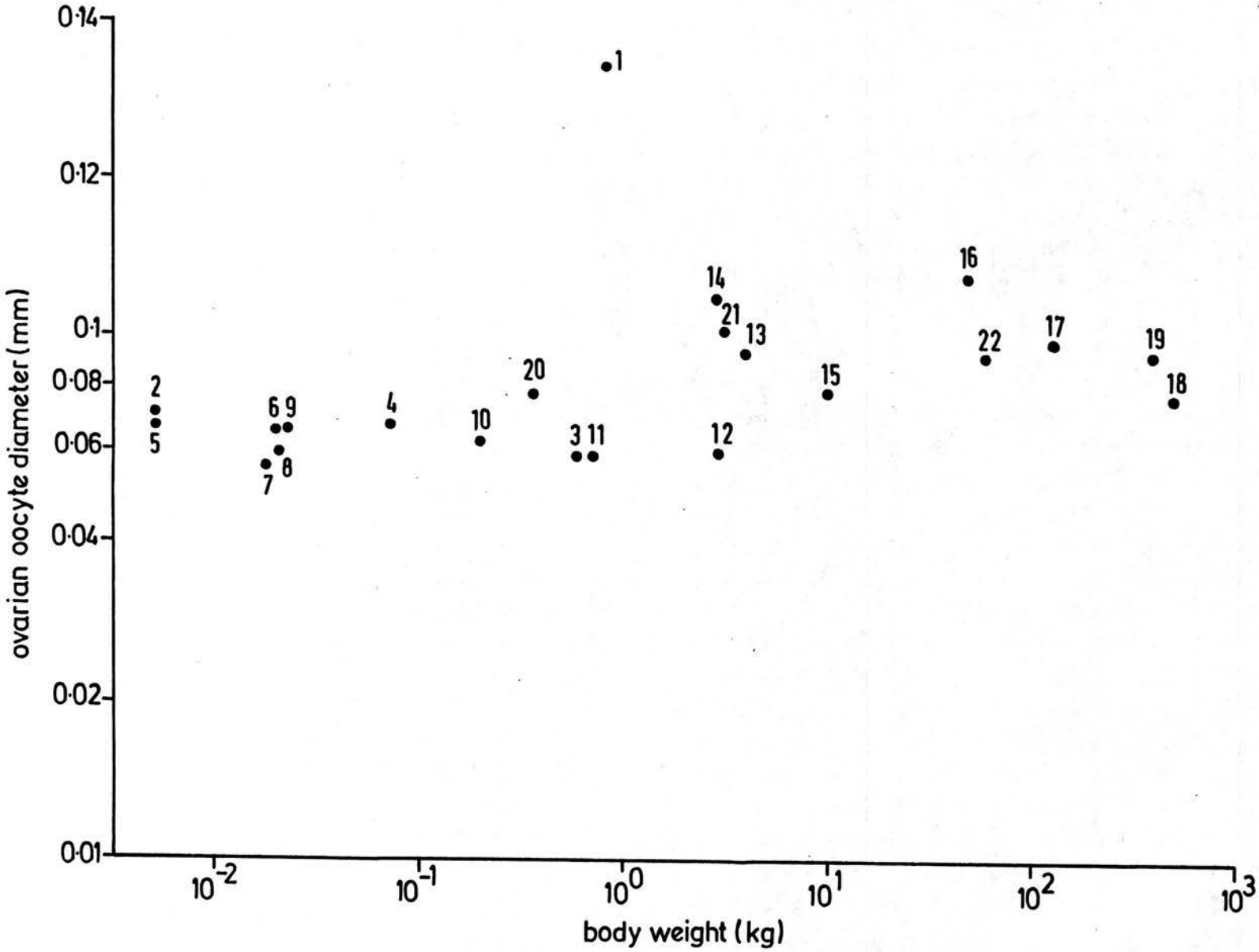
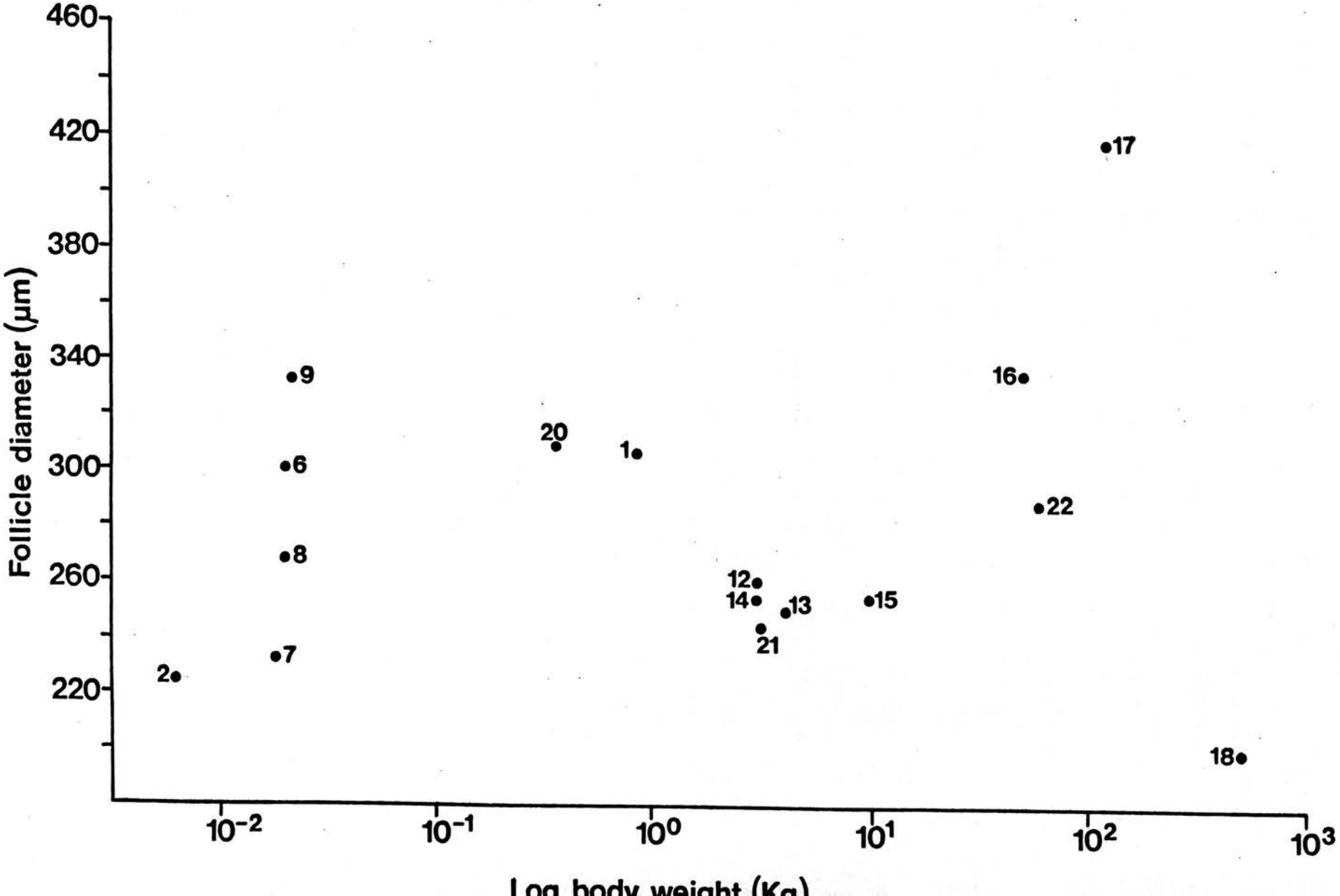

0·14
0·12
0·1
0·08
0·06
0·04
0·02
0·01
ovarian oocyte diameter (mm)
1
2
3 11
4
5
6 9
7
8
10
12
13
14
15
16
17
18
19
20
21
22
10^{-2}
10^{-1}
10^{0}
10^{1}
10^{2}
10^{3}
body weight (kg)

Figure 6.3:

Relationship between size of follicle in microns at onset of antrum formation and body weight on log coordinates for 16 species.

A regression line was not appropriate for these data.


460
420
380
340
300
260
220
Follicle diameter (μm)
9
6
8
7
2
20
1
12
14
13
21
15
17
16
22
18
10^{-2}
10^{-1}
10^{0}
10^{1}
10^{2}
10^{3}
Log body weight (Kg)

Figures 6.4 & 6.5 show the allometric relationship between individual and total Graafian follicle surface area and body weight. The line of best fit and 95% confidence limits have been calculated. The allometric formula for individual Graafian follicle surface area = $(y = 11.4M^{0.81})$ and for total Graafian follicle surface area $(y = 58.4M^{0.65})$.

<u>Graafian follicle volume : body weight</u>:

As was expected individual and total Graafian follicle volumeSwere correlated significantly with body weight yielding correlation coefficients of 0.869 and 0.921 respectively. Figures 6.6 & 6.7 show the allometric relationship between individual and total volumes with body weight. The allometric formula for individual Graafian volume = $(y = 3.61\ M^{1.21}$ and 1.056 and for total Graafian follicle volume = $(y = 18.5\ M^{1.06})$ with this exponent not being significantly different from unity.

In all of the species examined mature follicles had a follicular antrum. In those mature follicles that exceeded 0.4mm diameter follicular fluid contributed more than 50% of the volume with this percentage being greater in larger follicles.

The epithelium of mural granulosa cells was 3-9 cell layers thick, without any systematic variation according to follicular diameter. The theca layers were more difficult to define; however, it was a general impression that larger follicles had more theca layers.

Figure 6.4:

Allometric relationship between the surface area of individual Graafian follicles and body weight.

Regression line with 95% confidence limits and allometric formula are given.

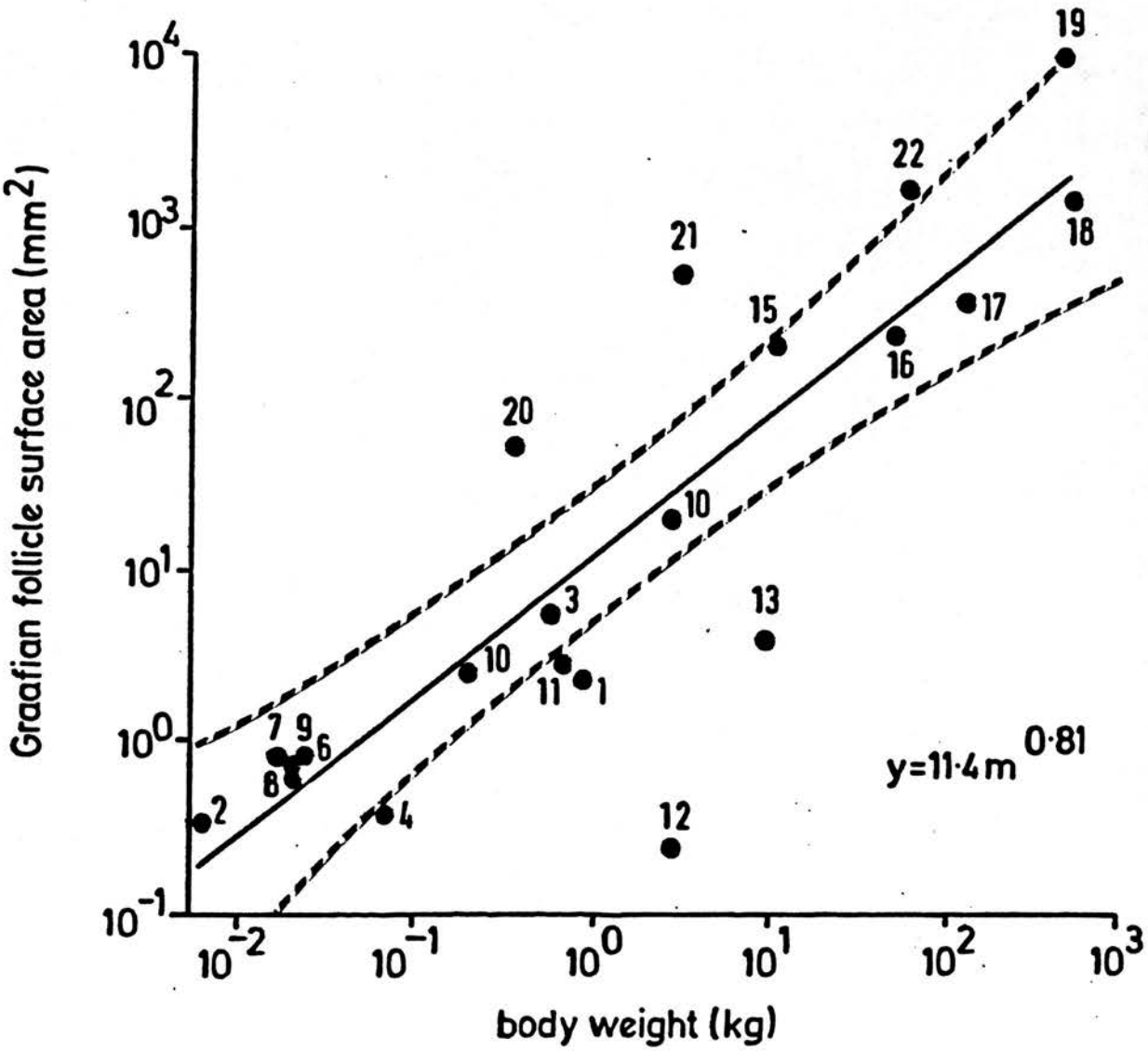


Figure 6.5:

Allometric relationship between the surface area of the ovulatory quota of Graafian follicles and body weight.

Regression line with 95% confidence limits and allometric formula are given.

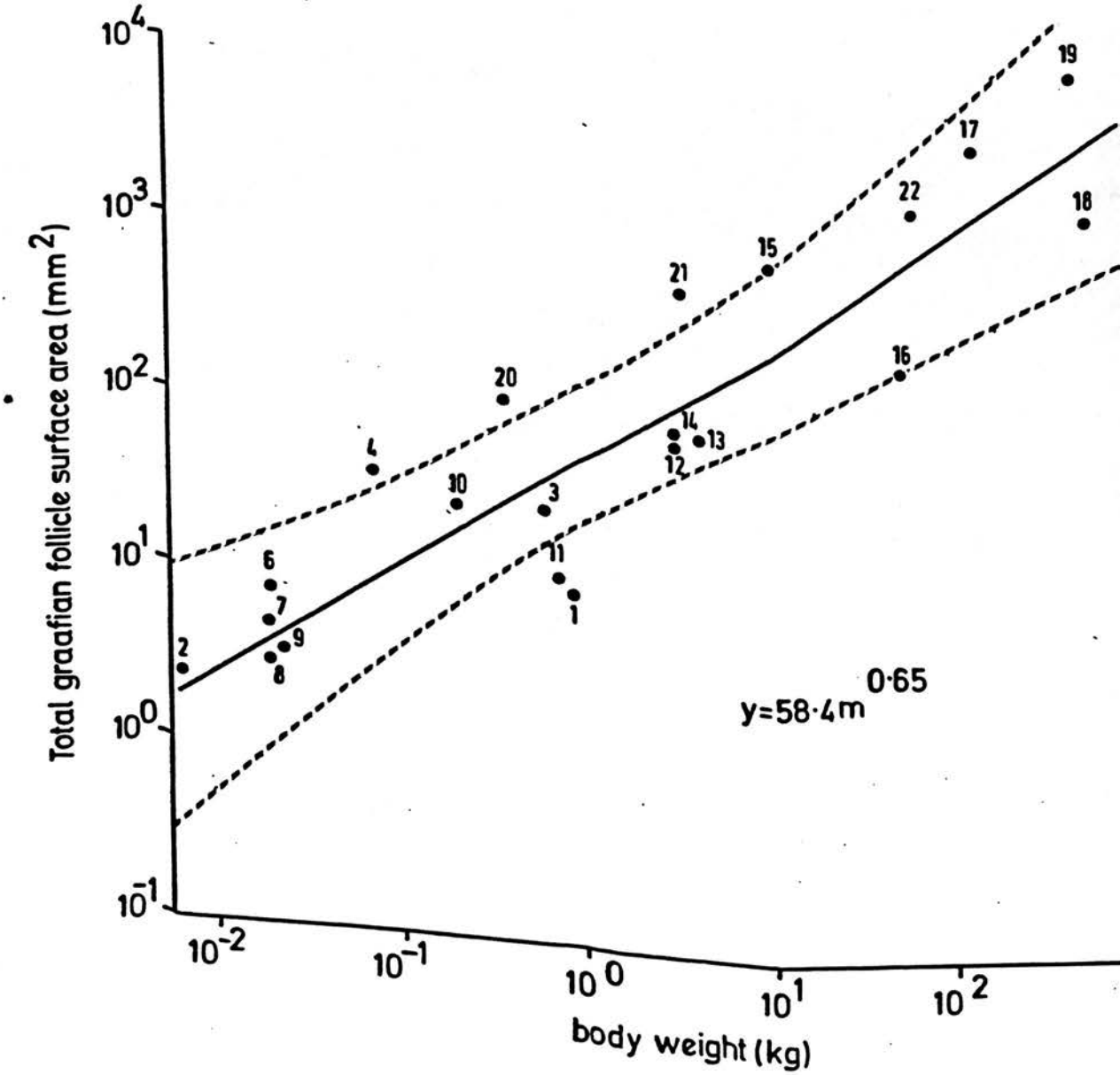


Figure 6.6:

Allometric relationship between the volume of individual Graafian follicles and body weight.

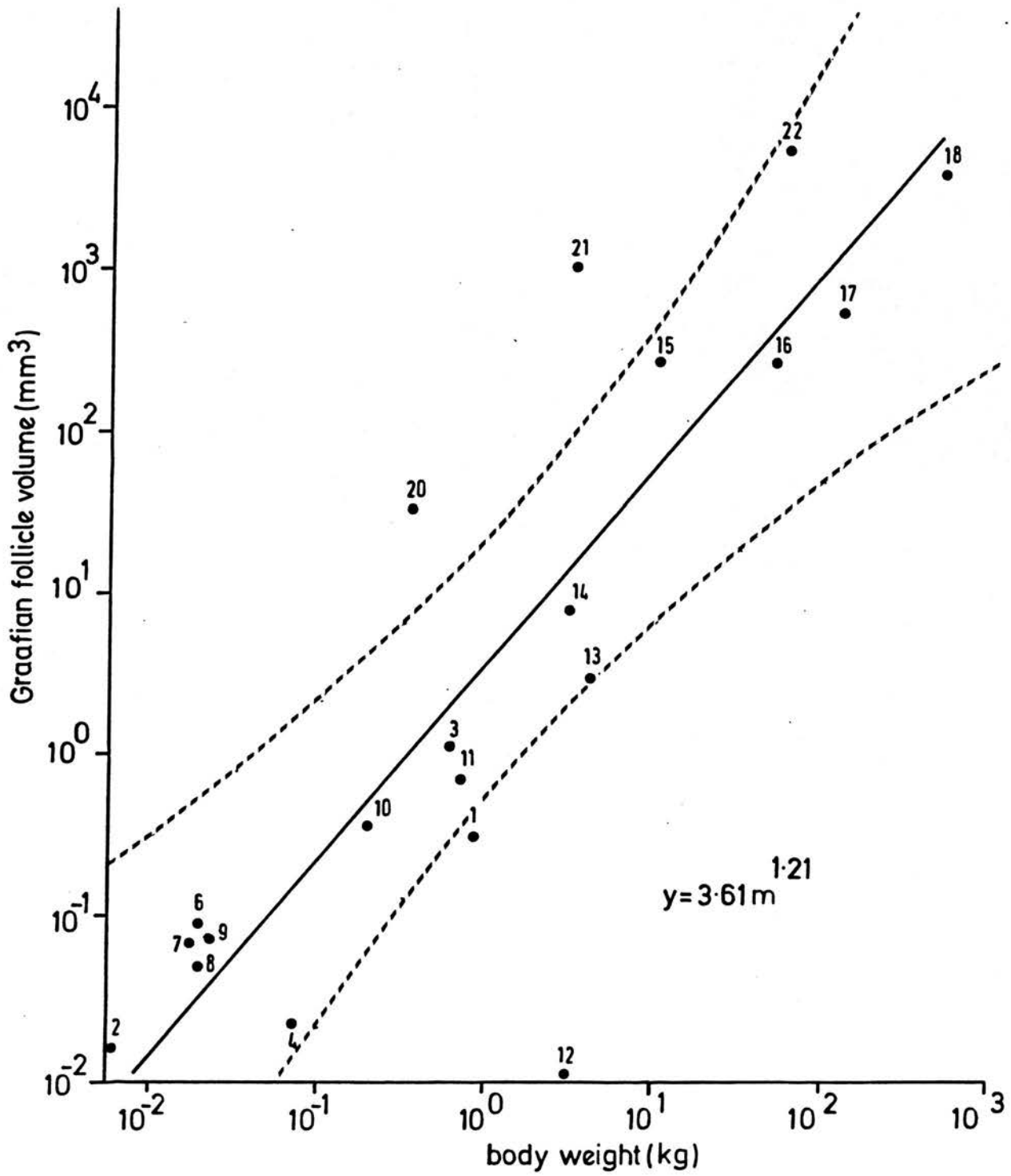
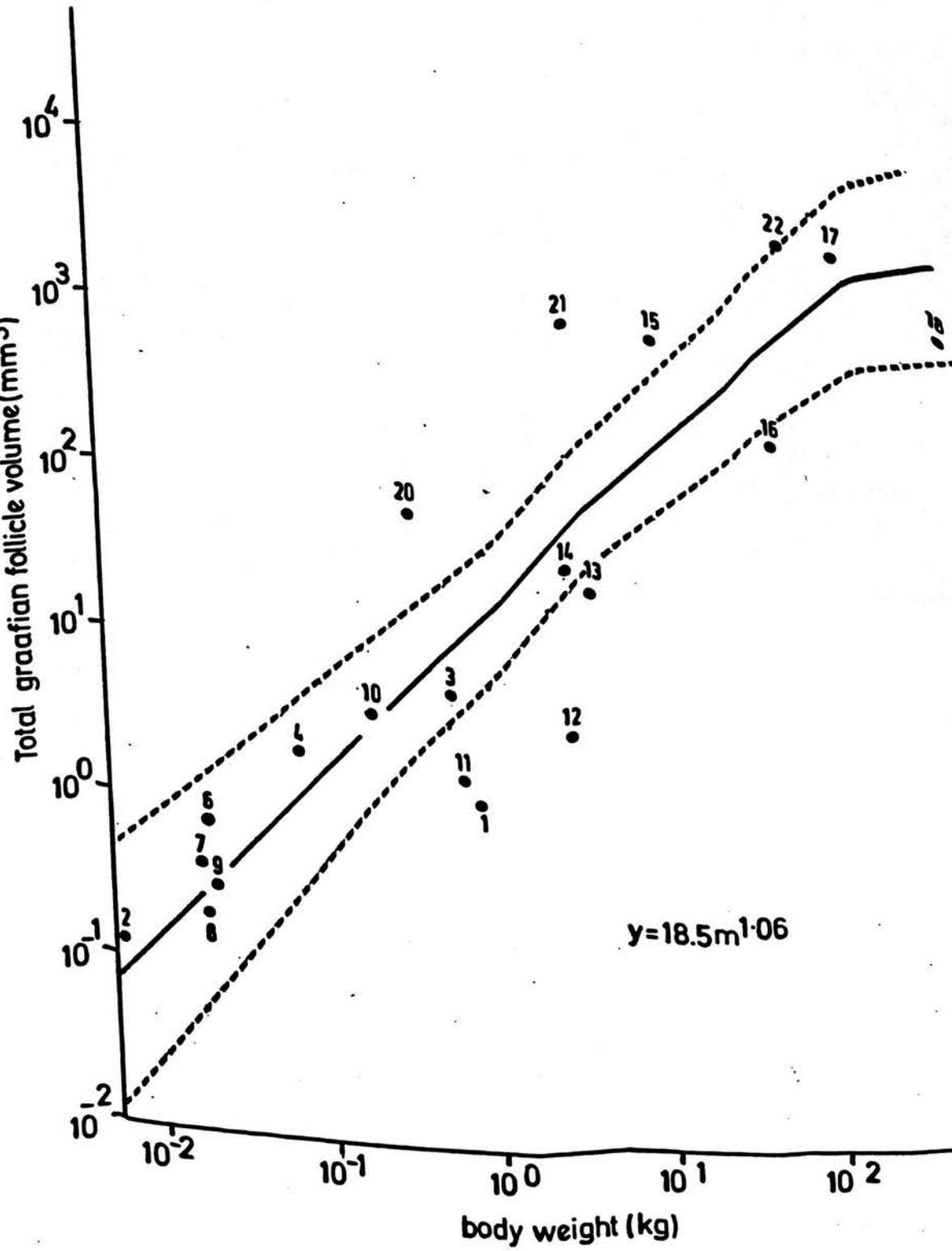


Figure 6.7:

Allometric relationship between the volume of the ovulatory quota of Graafian follicles and body weight.



The dimensions (diameter, surface area & volume) of Graafian follicles ranged from 2-5 orders of magnitude above the lowest values, which were observed in the shrew and plains viscacha. Body weight differences could account for most of the variation in Graafian follicle sizes; however, greater statistically significant correlation coefficients were obtained when the number of ovulatory follicles was considered and dimensions obtained for this total number.

Section two:

Number of primordial follicles: body weight:

The mean numbers of primordial and growing follicles obtained for 19 species are presented in Table 6.4. The numbers of primordial follicles at the onset of reproductive life correlated significantly with body weight, yielding a correlation coefficient (r) of 0.953 (see Table 6.3). Most interspecific variation in numbers of follicles could be accounted for on the basis of body weight differences rather than in numbers or frequency of ovulation or by reproductive pattern. It was not possible to obtain follicle counts for those animals with extraordinarily high ovulation rates, i.e. plains viscacha and elephant shrew, as there were no serial sections available for these species.

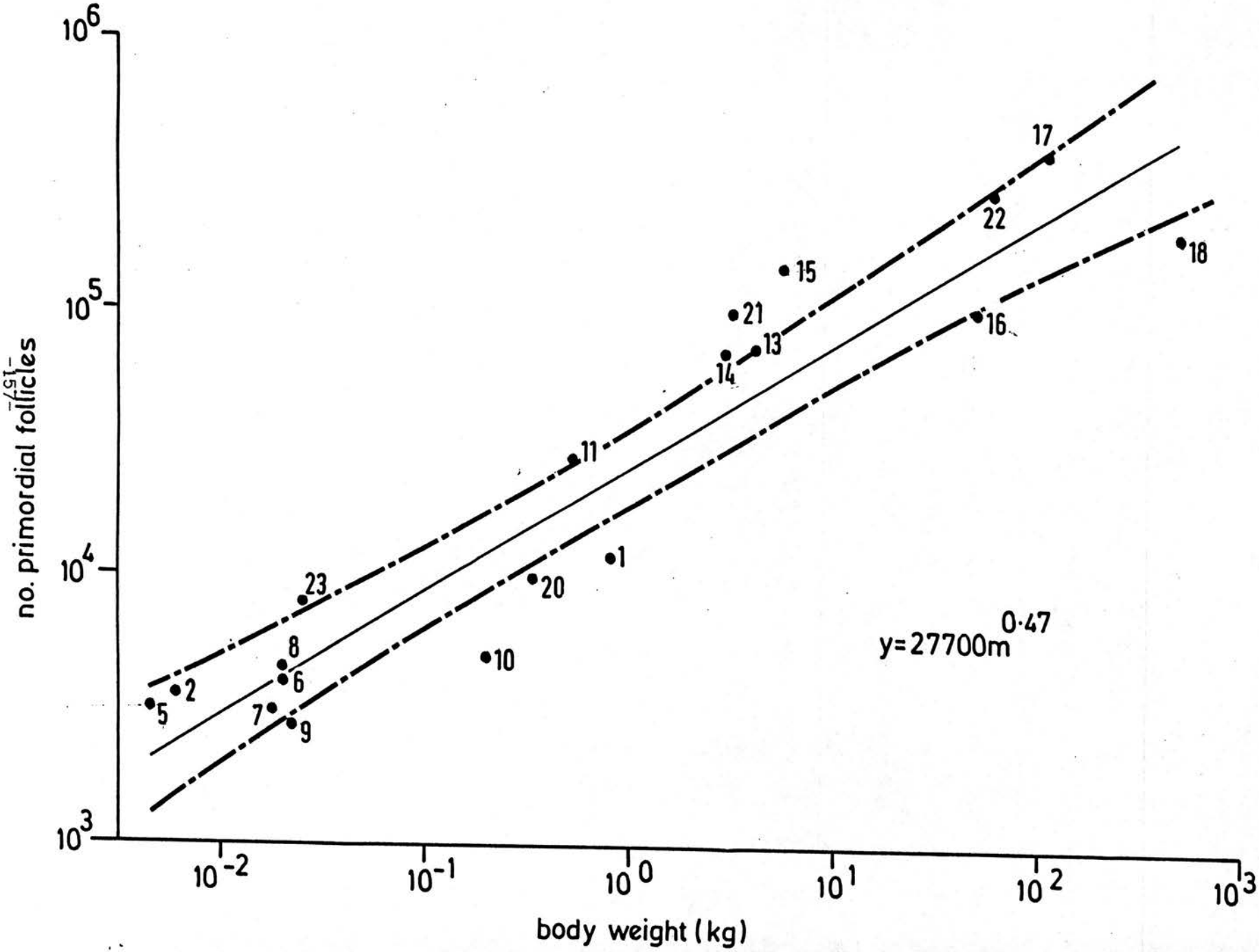
Figure 6.8 shows the plot of the number of primordial follicles at the onset of reproductive life with body weight in 19 species. The species characteristic number of follicles at the onset of reproductive life varied hypoallometrically with a body weight exponent of 0.475+/- 0.038.

Table 6.4: Mean number of primordial and growing follicles.

Species	No. primordial follicles	No. growing follicles	Growing fraction
Bandicoot	12440	915	0.049
Common shrew	3560	-	-
Pipistrelle bat	3268	254	0.072
Greater horseshoe bat	7950	-	-
House mouse	4270	676	0.165
Wood mouse	3170	164	0.032
Bank vole	4380	269	0.056
Field vole	2858	179	0.050
Norway rat	5180	-	-
Guinea pig	29200	-	-
European rabbit	75120	3222	0.040
Domestic cat	74520	1132	0.021
Domestic dog	150380	29775	0.198
Sheep	105450	475	0.007
Swine	420000	-	-
Cattle	210000	-	-
Common marmoset	17220	1877	0.109
Rhesus monkey	100000	16100	0.161
Human	302000	12090	0.040

Figure 6.8:

The number of primordial follicles at the onset of reproductive life in relation to body weight for 19 species (see table 6.1 for species codes). The relationship is depicted by linear regression analysis and 95% confidence limits are shown. The allometric formula describing this relationship is also shown.


no. primordial follicles
body weight (kg)
y=27700m^0·47
10^6
10^5
10^4
10^3
10^-2
10^-1
10^0
10^1
10^2
10^3
1
2
5
6
7
8
9
10
11
13
14
15
16
17
18
20
21
22
23

These variables were highly correlated with a correlation coefficient of 0.91 indicating that most interspecific variation can be accounted for by differences in weight rather than numbers of ovulations or reproductive pattern.

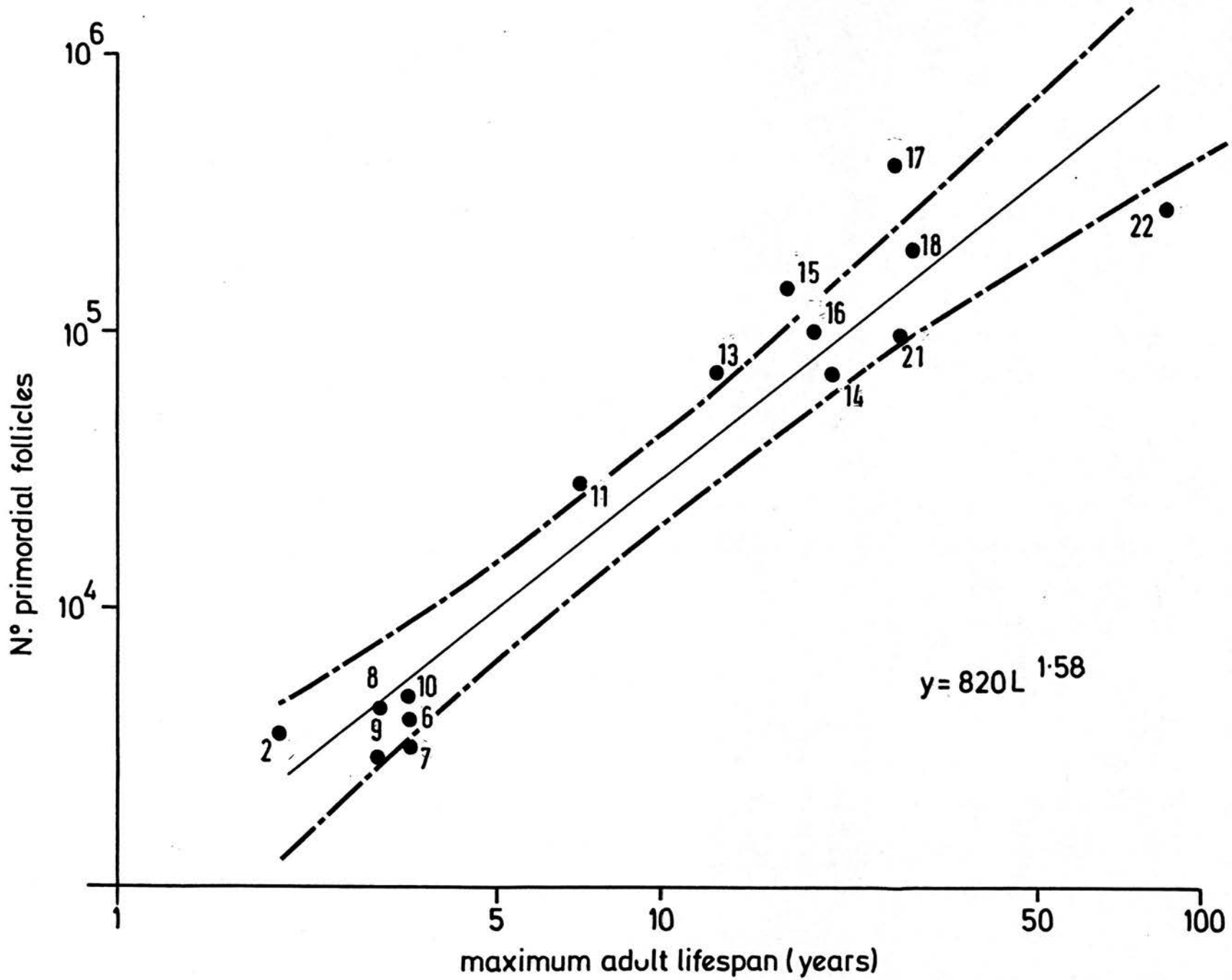
Number of follicles : maximum longevity:

The number of follicles in 19 species at the onset of fertile life varied hyperallometrically with an exponent of 1.58 (see Fig. 6.9). The correlation coefficient obtained for the plot was statistically significant with an r value of 0.90.

Figure 6.9:

The relationship between the length of the maximum expected lifespan and the total numbers of primordial follicles per individual at the onset of reproductive life.

Data has been obtained for fifteen species. Linear regression analysis with 95% confidence limits is depicted as is the allometric formula.


N° primordial follicles
10^6
10^5
10^4
1
5
10
50
100
maximum adult lifespan (years)
y= 820L 1·58
2
8
10
9
6
7
11
13
15
16
17
18
14
21
22

Discussion:

To observe patterns in ovarian structure between species allometric analysis has been utilised. This approach is primarily used for describing interspecific variation; deviations from any general pattern can help to reveal principles and connections that might otherwise have been overlooked. The allometric equation has been used to express biological relationships from as early as 1891 when Snell compared brain size with body size. Studies on scaling of components of the reproductive system have been neglected (Calder, 1984). This study looked at the scaling relationships of a number of reproductive variables in a wide range of taxonomic groups. The results have shown that the differences in sizes of Graafian and primordial follicles between species can be predicted by the differences in adult body weight. The scaling relationships follow the general allometric formula, $y = aM^{b}$. The value of this type of analysis for describing a relationship between two variables and for predicting one variable from another which has been measured rests on several assumptions, these and possible physiological explanations for the relationships obtained will be discussed.

The size of oocytes of eutherian mammals did not show any relationship with body size as they were all remarkably similar, this observation supports the results of earlier studies (Austin, 1976) but is in contrast with spermatozoa which are scaled negatively with body weight (Cummins & Woodall, 1985). The only representative of the marsupials had the largest oocyte and this confirms other studies in which the oocyte of the marsupial, *Dasyrus viverrines* had a value of 250μm (Austin, 1976).

The logarithms of the linear dimensions of Graafian follicles with respect to body weight produced highly significant statistical correlations and marginally significant correlations were obtained for the plot of primordial follicle diameter and body weight. It may seem that the allometric relationship observed for Graafian follicles could be explained on the basis of a scaling up from that observed among primordial follicles, but this cannot be possible since the development of different compartments are involved in the two types, namely the ooplasm and the antrum. Allometric variation of interspecific Graafian follicle size would appear to be due to variation in the volume of follicular fluid as the thickness of the *membrana granulosa* was relatively constant between species. Primordial follicle size was significantly correlated with several of the other variables but any physiological basis for scaling of primordial follicle sizes is difficult to postulate thus, it is unlikely that there is a causal relationship between these two variables.

Graafian follicles are integrated in development for ovulation and for the secretion of hormones into the body pool and so it might be expected that the total dimensions of the preovulatory set of follicles characteristic for each species would be better correlated with body weight than individual dimensions. The correlation coefficients for Graafian follicles were substantially increased by calculating the dimensions on the basis of the mean ovulatory quota for the species. The importance of considering the ovulation number in the calculation of surface area and volume is highlighted by the fact that the outlying values of polyovulating

species were constrained after this manoeuvre. Since a species such as plains viscacha can ovulate on average 300 oocytes, it is expected that any scaling effect would be on the basis of this total number of follicles.

The regression line obtained from the plot of body mass against Graafian size would predict that the Graafian volumes of the plains viscacha (Lagostomus maximus) and the Elephant shrew (Elephantulus myurus), would be 22.0 and 0.23mm^3 instead of the observed values of 0.01 and 0.02mm^3. Since the ovulation rates of these species vary from 200-800 and from 50-180,respectively (Weir, 1971; Tripp, 1971) this must be taken into consideration as the ovarian mass and surface area required for accommodating and ovulating the predicted follicle sizes in such large numbers may not be physiologically realistic. Further information is required to determine whether an interactive effect of numbers and sizes of preovulatory follicles exists continuosly across a wide range of species, or is confined to those species with exceptionally high ovulation rates where the importance of scaling is obvious.

The species of bat from which measurements were obtained, the lesser horseshoe bat (*Rhinolopus hipposideros*), does not follow the pattern of Graafian follicle scaling. The ovaries of this bat contains one or two Graafian follicles which measure 0.3mm in diameter (Harrison Matthews, 1937). The follicular volume in this bat, although similar to that of terrestrial mammals of a comparable size (e.g. common shrew), is considerably less than that predicted by allometry for the known ovulatory quota. This is

however, based on limited information and so conclusions have to be tentatively drawn.

Variation in Graafian size might also be explained on the basis that it is the maximum dimensions of these structures that determine the size of the corpora lutea. Corpora lutea are vascular and more solid than follicles and so they might be expected to vary isometrically with the volume for hormone distribution, this relationship cannot, as always, be explained so simply as in some species there are accessory corpora lutea and heterogeneous populations of luteal cells (Schwall & Niswender, 1985).

A relationship between the total surface area of a preovulatory set of follicles and the distribution volume (i.e. extracellular fluid) for oestrogen and other follicular hormones is not supported by the present results. Plasma oestrogen levels are independent of body weight, the isometric variation between the volumes of Graafian follicles and body weight, of which the hormone distribution volume is a fixed proportion (Stahl, 1967), would be expected if follicles consisted entirely of a homogeneous population of secretory cells. The majority of space in follicles of most species is extracellular and there is only a shell of theca and granulosa cells with most of the metabolic activity being confined to the periphery of the structure (Gosden & Byatt-Smith, 1986). Isometric variation is unexpected, unless the follicular fluid, which contains high concentrations of steroid hormones (Edwards, 1974), acts as a hormone store and is dynamically involved in the maintenance of circulating hormone levels.

Allometric relationships as well as providing general descriptions of scaling relationships, can draw attention to exceptional data and these may indicate peculiar physiological or anatomical features. In primates, the disproportionately large size of Graafian follicles is of particular interest when considered in relation to the high levels of oestradiol 17β found during the preovulatory phase. The levels are about 3-10 fold greater than those in non-primate species which are invariably <0.5 pmol ml^{-1} (e.g. for non-primates: Scaramuzzi et al., 1970; Noden et al., 1975; Smith et al.,1975; Austad et al., 1976; Hodges et al., 1983; and for primates: Weick et al., 1973; Korenman et al., 1974; Reyes et al., 1975; Nadler et al., 1979). Such differences among the other gonadal steroids have not been observed in either males or females. That these high levels distinguish those species having menstrual cycles and those which do not can be discounted since especially high levels of oestradiol are present among the New World monkeys, which do not menstruate (Wolf et al., 1977; Harlow et al.,1984). The elevated oestrogen levels in primates could be due to larger follicles, and it is also possible that they reflect a slower rate of clearance from the circulation resulting from greater plasma sex steroid binding activity and consequent protection from metabolism (Corvol & Bardin, 1973; Siiteri et al., 1982).

When body size correlations and dependencies are being studied, it is crucial to avoid the mixing of phylogenetic and ontogenetic data (Calder, 1984). This study was concerned with inter rather than intraspecific differences in body weight and associated

correlations. The samples obtained for any given species showed little difference in body weight. Allometric relationships of variables across a range of species do not necessarily apply within a particular species (Calder, 1984), and this can be illustrated in relation to ovarian follicles in sheep. The mean ovulation rate of the Merino breed of sheep is 1.2 with Graafian follicles measuring 8mm whereas in the highly fecund Booroola breed the values are 5.2 and 4mm (Driancourt et al., 1985). The total Graafian surface areas and volumes in the two species are therefore 241 and 261 mm^2 and 321 and 174 mm^3, respectively, in the two breeds. These findings contradict the expectation that the total volumes should be the same. In this situation it must always be remembered that other factors are interacting since these species have been selectively bred for several generations for desirable characteristics.

The species characteristic number of follicles per individual at the onset of reproductive life varied hypoallometrically with body weight and most interspecific variation was accounted for by differences in adult body weight rather than in numbers or frequency of ovulation or of reproductive pattern. The range of species looked at with respect to the number of follicles did not include those species with exceptionally high ovulation rates and it is hoped that in the future suitable data will be available to compare the number of small follicles on a body weight basis in the plains viscacha (*Lagostomus maximus*) and the elephant shrew (*Elephantulus myurus*). This allometric relationship for the number of follicles can also be read as for oocytes since in all species (excepting the bitch, see chapter 7) polyovular follicles were

rarely observed. This is in contrast with observations on the primate testis which varies in size and so gametogenic activity according to reproductive behaviour and body weight (Harcourt *et al.*, 1981).

The allometric relationship between the maximum longevity of a species (L, in years) and body weight has been calculated to be $11.8M^{0.20}$ (Sacher, 1959) this result corresponds with that estimated for the adult lifespan (i.e. post-pubertal life) in this study, $9.85M^{0.25}$. A comparison of the allometric exponents of body weight and life expectation at puberty and the numbers of follicles with both body weight and longevity, indicates that the size of the follicle store rises more steeply per unit increase in body size than does life expectancy. This would suggest that follicular redundancy is greater in larger animals. Follicular redundancy is a feature of the ovarian system with most species losing large numbers of follicles prior to the onset of reproductive life. Follicular redundancy can occur in other forms as for example in the greater horse-shoe bat and the south american rodent, Lagidium pervanum in which ovulations only occur from the right ovary (Harrison Matthews, 1937; Pearson, 1949).

This excess of follicles in relation to requirements in most species would appear to provide a wide margin of physiological safety, which has been postulated for other phenotypic characters (Gans, 1979). Whether the formation and utilisation of follicles is under strict genetic control is not known. The increased number of follicles observed in larger species could be an important

adaptation for a greater span of adult life. Species differences in the number of oogonial mitoses during the pre- or perinatal period and differences in the rates of germ cell wastage could contribute to the pattern at puberty. Larger animals have a larger store of follicles at the onset of reproductive life than smaller animals but this may reflect differences in post-natal utilisation of follicles. Germ cell numbers throughout reproductive life are controlled by a combination of growth from the primordial pool of follicles and atresia. Data on follicle utilisation from which to make interspecific comparisons and further studies are required to determine whether a balance exists between the size of the store and the rate of utilisation, leading to similar residual numbers at the end of life.

The numbers of follicles and the dynamics of their utilisation must ensure that individuals of the species are provided with sufficient numbers of germ cells throughout their reproductive life. Primary ovarian failure has been rarely reported to occur before the end of life in animals, except among a few inbred and mutant strains of rodents (Finch & Gosden, 1986). The main exception to this is of course the menopause in human females. The menopause usually occurs around mid-life and it is thought to be mainly as a result of follicular deficiency (Gosden, 1985b). The estimates obtained in this study indicate that the size of the human follicular population at puberty is as predicted by species body weight. There does not seem to be a deficiency at this age in our species. This ovarian failure in humans could be as a result of excessively high rates of follicle deaths, however, no evidence can be presented to

support this. It could be argued that menopause has arisen adventitiously during evolution as a by-product of the extension of lifespan beyond expectation based on body size as allometry predicts a lifespan of around 30 years for humans in contrast with the observed maximum of 100 years.

By studying the relationships of two variables we are looking at a very simplified model of the system. Statistical correlations obtained for two variables do not necessarily mean biologically significant relationships or causality. Correlations obtained between two factors may be influenced by a third factor and the potential for interrelationships must always be considered in this type of analysis. In the case of relationships between ovarian follicular utilisation and body weight the variabilty of life patterns are superimposed. For instance in the two species of bats studied, the numbers of follicles were commensurate with body weights but both species were outliers with respect to adult longevity which was based on limited information that the expected lifespan of these species in their natural environment is 10-20 years (Stebbings, 1977). Compared with rodents and insectivores of a similar size, these species presumably depend upon parsimonious utilisation of follicles in order to prevent premature loss of follicles. No observations exist to support this expectation but perhaps a lowering of metabolic rate during hibernation could provide one mechanism, if not the complete explanation.

The significance of this type of study is difficult to evaluate since we are relying heavily on several subjective elements. This

approach rests on the assumptions that the data are taken from a representative sample of species and of individuals and that measurements are reliable. The range of animals used in most studies depends very much on availability and this study is no exception. Inevitably this leads to selecting readily available species and results in a bias towards the domesticated species and laboratory stocks. This study has attempted to study as wide a range of species as possible by including representatives of twenty-three taxonomic groups but it is hoped that this will be extended with the inclusion of several other species.

To conclude; this study has used allometry to explore the scaling relationships between a number of variables relating to the ovary. This is a descriptive approach but physiological explanations can be tested, however, in the case of ovarian parameters it is difficult to obtain information from a wide range of species at appropriate ages to explore these. This approach contributes to our understanding of biological patterns and from the descriptions obtained we are able to identify and focus on the areas that most require explanation. It is unlikely that geometrical scaling factors are major influences upon follicular sizes, since many other variables must be considered. Isometric relationships have not been found among other endocrine organs (Brody, 1945; Harcourt et al., 1981), and there is no simple rule for the apportionment of organ space within the abdomen.

Chapter Seven:

Incidence of polyovular follicles in the domestic bitch.

Introduction

Ovarian follicles normally contain only a single oocyte, this is, however, not an absolute rule since follicles containing several oocytes have been found in many species. The pioneering work of Von Baer (1827) which led to the first correct description of the mammalian oocyte noted the presence of polyovular follicles and subsequent work has demonstrated these follicles occur in a wide range of species; The review by Hartman (1926) comprehensively covers the literature on this subject up to 1925/26. However, although there is a considerable literature dealing with this topic there are still many fundamental questions regarding the origin, significance and developmental fate of polyovular follicles remaining to be tackled.

The majority of those who published studies on polyovular follicles were satisfied to merely record their presence during the course of a histological survey and as a result polyovular follicles have been reported in a wide range of species (see Hartman, 1926 and Table 7.1). Many of these studies were, however, based on a single observation (O'Donoghue, 1912) and sometimes after the examination of only a single histological section, (e.g. Kennedy, 1924). There has been no standardisation of methodology or classification of follicle types and because there is a dearth of quantitative data the frequency of polyovular follicles can be cited for few species. Since Hartman reviewed the topic in 1926 there have been several additions to the species in which polyovular follicles have been found (see Table 7.1) but there remains a lack of standardisation between studies which makes quantitative inter-specific comparisons impossible.

Table 7.1:
List of Species in which polyovular follicles have been found and references since 1926.

Order	Generic name	common name	Reference.
Carnivora	*Canis familiaris*	Bitch	Ota, T. 1934.
	Felis catus	Cat	Dederer, P.H., 1934 Shehata, R., 1974.
	Mustela putorius	ferret	Mainland, D., 1927.
	Mephitis mephitis	striped skunk	Leach, & Conway, 1963
Rodentia	Rattus norvegicus	Rat	Lane, C.E., 1938. Davis, & Hall, 1950 Dawson, 1951 Kent, 1962a
	Mus musculus	mouse	Engle, E.T., 1927. Fekete, E., 1950. Kent, H.A., 1960. Iguchi, T., 1986.
	Cavia porcellus	guinea pig	Collins, D.C. & Kent, H.A. 1964
	Mesocricetus auratus	golden hamster	Kent, H.A., 1958; 1962b; 1964 Kent & Mandel, 1968; 1970 Bodemer&Warnick, 1961a; 1961b Zybina, E. &Grishche, T. (1980)
	Sciuridae	prairie dog	Stockard, A.H., 1937.
Lagomorpha	*Oryctolagus cuniculus*	rabbit	Desaive, P. 1949. Marois & Buvet, 1972. Szöllösi, 1978. Al Mufti *et al.*, 1987.
Artiodactyl	*Capra hircus*	goat	Harrison, R.J., 1948
Primates	*Lemur macaco*	lemur	Harrison, R.J., 1949.
	Scumiri sciurea	squirrel monkey	Harrison, R.J., 1949 Graham & Bradley, 1971
	Maccacus rhesus	rhesus monkey	Lloyd & Rubenstein, 1941.
	Homo sapiens	man	Pankratz, D.S., 1938 Bacsich, P., 1949. Curtis, E.M., 1962. Jones, G.S., 1968. Papadaki, L., 1978. Gougeon, A., 1981.

Table 7.1: (cont).

Order	Generic name	common name	Reference.
Marsupiala	*Potorous tridactylus*	potoroo	Ullmann & Dairi, 1986
Cetacea	*Balaenoptera borealis*	sei whale	Best & Bannister, 1963
Chiroptera	*Myotus lycifigus*	bat	Guthrie, J., 1938.

Interest in polyovular follicles has stemmed from two questions, namely, their ontogenesis and their contribution to fecundity. These follicles have frequently been regarded as pathological entities and this has led many investigators to pay attention to the incidence of oocyte death and the role of sex steroid and gonadotrophin levels (Lane, 1938; Bodemer & Warnick, 1961a; Kent & Mandel, 1970). These early enquiries were, however, influenced by a limited knowledge of reproductive endocrinology and the widespread but erroneous belief that oocytes are formed continually throughout life.

The present study was devised with several purposes in mind:

(1) To obtain quantitative information on the incidence of polyovular follicles in the domestic bitch in successive stages of follicular development and to compare this in both young and old animals.

(2) To asses the contribution of polyovular follicles to the growing population of follicles,

(3) To make morphometric comparisons of uniovular and polyovular follicles throughout follicular development,

(4) To evaluate the developmental potential of the oocytes within polyovular follicles.

The objectives of this study required the availability of ovaries having a sufficiently high incidence of polyovular follicles in order to obtain reliable data. Since published reports rarely present numerical data and, where these exist, they are not based on comparable classification of follicles, a survey was carried out to identify a suitable species for detailed study. The domestic bitch was chosen since this species

had the highest frequency of polyovular follicles than any of the other 14 species looked at.

The domestic bitch (*Canis familiaris*) was one of the first species in which polyovular follicles were recorded (Von Baer 1827) and the bitch is often quoted with the opossum as a species in which these structures are common (Ota, 1934; Brambell, 1956; Harrison & Weir, 1977). Many of the studies following on from Von Baer's observations concluded that polyovular follicles although abundant in the immature bitch were rare in the adult suggesting that these structures were more susceptible to death in early stages.

Materials and Methods:

Incidence of polyovular follicles in different species:

Histological specimens from a minimum of four young adult individuals representing fifteen species were obtained in toto, with the exception of humans where only two specimens were available (see Table 7.2). The proportions of growing follicles containing 2, 3, 4 or more oocytes were estimated and a minimum of 1000 follicles were examined in each case. The nucleolus of the oocyte was used as a marker for counting the follicles and when appropriate, adjacent sections were searched to find the complete set of oocytes in polyovular follicles.

On the basis of the findings, additional specimens were obtained from bitches for further study.

Table 7.2: Frequency of polyovular follicles in 16 mammalian species.

Species	% of growing follicles containing the understated number of oocytes		
	2	3	4+
Bandicoot	<0.10	<0.10	<0.10
Common shrew	<0.10	<0.10	<0.10
Pipistrelle bat	<0.10	<0.10	<0.10
Greater horseshoe bat	<0.10	<0.10	<0.10
House mouse	<0.10	<0.10	<0.10
Wood mouse	<0.10	<0.10	<0.10
Bank vole	<0.10	<0.10	<0.10
Field vole	<0.10	<0.10	<0.10
Norway rat	<0.10	<0.10	<0.10
European rabbit	0.91	<0.10	<0.10
Domestic cat	3.61	0.45	<0.10
Domestic dog	8.89	2.97	2.08
Sheep	<0.10	<0.10	<0.10
Common marmoset	<0.10	<0.10	<0.10
Rhesus monkey	1.49	0.30	<0.10
Human	2.72	0.19	<0.10

Source of Specimens:

Ovaries were collected from virgin cross-bred bitches during routine veterinary spaying from the Department of Animal Surgery, University of Edinburgh. Two groups were obtained on the basis of age of the animals: Group one consisted of ten bitches aged from 1 - 2 years old, with specimens always being collected during anoestrus and usually following the first or second oestrus. Group two consisted of five anoestrous bitches aged between 7-11 years. None of the animals were being spayed for reasons of disease. Details of weight, age and previous history were also obtained (see Table 7.3).

Histology:

The upper part of the reproductive tract including the ovaries was fixed in buffered formalin immediately following excision and held in this fluid during transportation. Within three hours of excision the ovaries were dissected from the upper tract and fixed in Susa fixative for 24 hours, dehydrated in graded alcohol for 6 hours and cleared in Toluene for 90 minutes. The cleared ovaries were embedded singly in paraffin wax. One ovary from each pair was taken at random and serially sectioned at 10μ and stained with haematoxylin and eosin.

Classification of follicles:

During follicular development there are many morphological changes as the oocyte and the surrounding cells differentiate. Schemes have been devised to classify follicles according to stage of development, these being based upon the size of the oocyte and size of the follicle as defined by the number of surrounding cell layers. In this study follicles were classified according to the scheme of Mandl & Zuckerman

(1951) (as described below, see Figure 7.1), this is based on the number of granulosa cell layers surrounding the oocyte in the largest cross section.

Stage I One layer of squamous or rounded granulosa cells with a small oocyte.

Stage II One continuous layer of cuboidal granulosa cells.

StageIII Two layers of cuboidal cells.

Stage IV Three layers of cuboidal cells.

Stage V Four or more layers of cuboidal cells.

Follicles were further classified into five categories according to the number of oocytes contained within them.

Type 1 Follicles with only one oocyte present.

2 Follicles containing two oocytes

3 Follicles containing three oocytes

4 Follicles containing four oocytes

>4 follicles containing more than four oocytes

So a follicle with two complete layers of cuboidal granulosa cells with two oocytes enclosed would be categorised as a stage III type 2 follicle. This convention will be used in the following sections.

Table 7.3:
Details of animals used in this study.

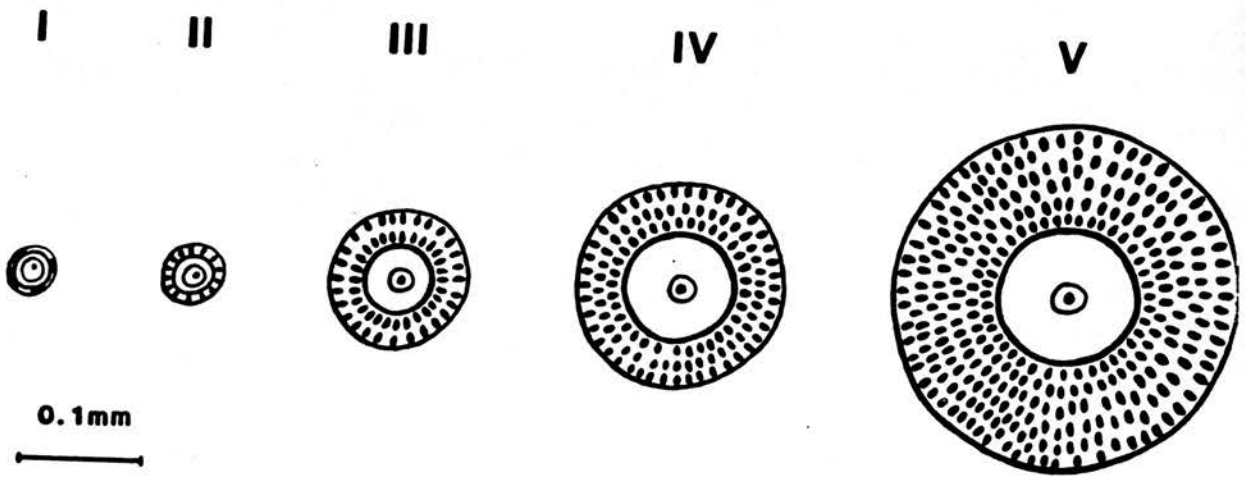
Group one:.....Group two:

code	age (months)	weight (Kg)	code	age (months)	weight (Kg)
B4	15	13.2	B23	84	16.5
B5	12	15.0	B22	84	3.8
B6	12	16.0	B11	96	12.7
B7	12	10.6	B21	102	11.0
B16	13	16.0	B14	132	9.5
B17	16	17.0			
B19	16	19.2			
B20	18	11.5			
B15	20	21.5			
B18	24	20.5			

All bitches were cross bred and ovaries were obtained during routine veterinary spaying.

Figure 7.1:

Diagrammatic representation of preantral stages of follicular growth in bitch ovaries. The figure is drawn to scale and represents only the uniovular types of follicles.



COUNTING PROCEDURES.

Every 20th section was marked for subsequently carrying out follicle counts. The counting procedures involved two steps:

1) Primordial follicles (i.e. small non growing types) were counted in every 20th section under high power (Mag X1000).

2) Growing follicles, stages II - V and types 1 - >4, were counted in every 20th section once the primordial count had been obtained using a magnification of X200.

Uniovular follicles;

Where only one oocyte was observed the follicles were classified as type 1 follicles and categorised according to stage of development by the system of Mandl & Zuckerman (1951) (see above). A follicle was counted if the nucleolar marker was present in the section under observation. Once the counts were obtained they were multiplied by the sampling frequency to obtain an estimate of total numbers and then a correction factor was applied to compensate for over counting (Abercrombie 1946) from the equation.

$$\frac{\text{section thickness}}{\text{section thickness + nucleolus diameter}}$$

Polyovular follicles;

When a follicle containing more than one oocyte was observed in the section being sampled, it would be counted if the nucleolus of at least one of the oocytes was visible in that section. To determine the exact number of oocytes within the follicle and so assign it to a particular type, the follicle was followed forward and backward into the serial sections. This procedure was only necessary for follicles with

greater than three cell layers because in most cases all oocytes could be observed on the same plane.

The data obtained were for follicles with histologically normal oocytes. Follicles where the granulosa cell layer was showing signs of pyknosis and disrupted oocytes were counted as atretic follicles and were not included in the final count since it was not always possible to reliably classify atretic follicles according to stage and type.

The correction factor applied to uniovular follicles (Abercrombie, 1946) on the diameter of the marker being observed i.e in this case the nucleolus. Polyovular follicles have several markers depending upon the number of oocytes present and so the correction factor of Abercrombie could not be used. A modification of the Abercrombie type correction factor proved difficult to obtain because of the geometry of these structures thus it was decided to obtain an empirical correction factor for a particular stage of polyovular follicles, i.e. the absolute total counts polyovular follicles at that stage in several sections compared with the value obtained at every 20th section. An empirical correction factor was obtained for stage III polyovular follicles by counting every polyovular follicle at that stage in 200 consecutive sections. By dividing the total number obtained by the values from every 20th section, a correction factor was obtained.

Measurements of Follicles:

Uniovular and polyovular follicles at different stages were selected at random and several variables were measured.

(a) The minimum and maximum diameter of the largest follicle section (the section containing the nucleolus) was measured using an eye-piece micrometer on a Vickers M17 microscope at a magnification of X1000 to obtain the average follicle diameter. All Measurements were taken from the basement membrane which is well defined, rather than the boundary of the theca which is indistinct.

(b) The minimum and maximum oocyte diameters were obtained using an eye-piece micrometer and from a section in which the nucleolus was present. The minimum and maximum diameter of the oocyte nucleus was also obtained by the same method.

(c) The minimum and maximum nucleolar measurements were obtained using an image-shearing micro measurement instrument (Vickers Instruments, York). Since nucleolar size indicates RNA synthetic activity it is important to obtain accurate reproducible measurements. This device measures nucleolar diameters more precisely than is possible with other light microscope techniques (Swyte & Rosberry, 1977) with a reproducibility of within 2% (Russell, 1983).

Data Analysis:

As the total counts of follicles obtained were highly variable the data were treated as proportions of each follicle type at each stage of development. The means and standard deviations were obtained. Simple probabilities were estimated for each type of follicle by considering each follicle type as a proportion of the total population of growing

follicles. Product-moment correlation coefficients (r) were calculated for the oocyte-follicle size relationship in uniovular and polyovular follicles.

RESULTS:

Histological observations:

In both groups of animals discrete primordial follicles and clumps of primordial follicles (see Figure 7.2a,b,c) were observed in the ovarian cortex. Primordial follicles accounted for 89% and 47% of the total follicle population in young and old animals respectively (Table 7.4). Death at these stages was observed in both young and old groups. The clumps of primordial follicles could not be described with confidence as primordial polyovular follicles because granulosa cell boundaries were not clear since the optical definition of paraffin sections is not good enough for this purpose.

Growing follicles up to small antral stages were observed with antrum formation appearing when follicles had five or more cell layers. Growing follicles consisted of both uni and polyovular follicles with atresia being noted at all developmental stages. Atresia was characterised by a wrinkling of the oocyte and pyknotic granulosa cells, atretic follicles accounted for around 30% of follicles in the older group and 10% in the young group. There did not appear to be a difference in the incidence of atresia between uni and poly ovular follicles, however, this cannot be strictly quantified as atresia is difficult to score for in polyovular follicles as often it is so advanced that it is impossible to determine the exact number of oocytes originally enclosed within the healthy follicle.

Figure 7.2a:

Ovarian cortex of a young adult bitch showing clusters of primordial follicles. H & E. X160.

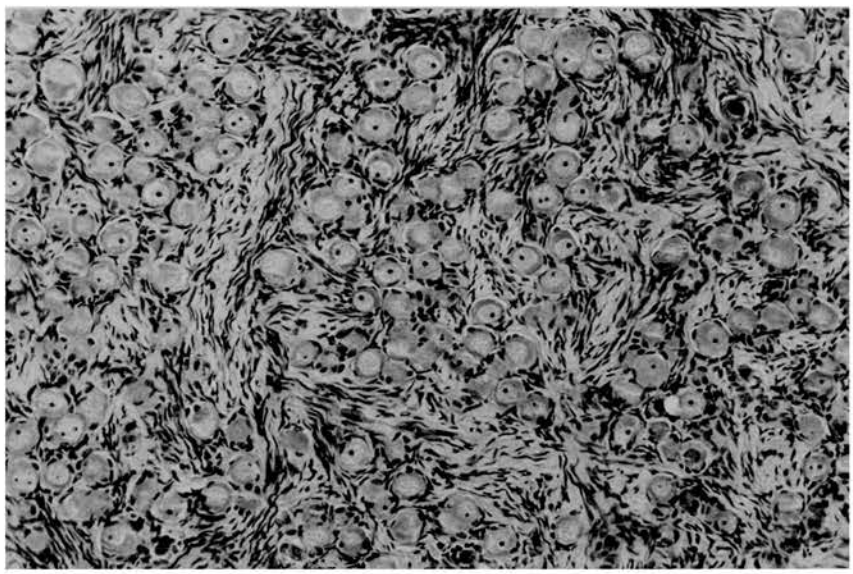


Figure 7.2b:

Cortex of bitch ovary showing primordial; uniovular and polyovular growing follicles and atretic follicles. A polyovular follicle containing 8 oocytes can be seen in this section. H & E. X160.

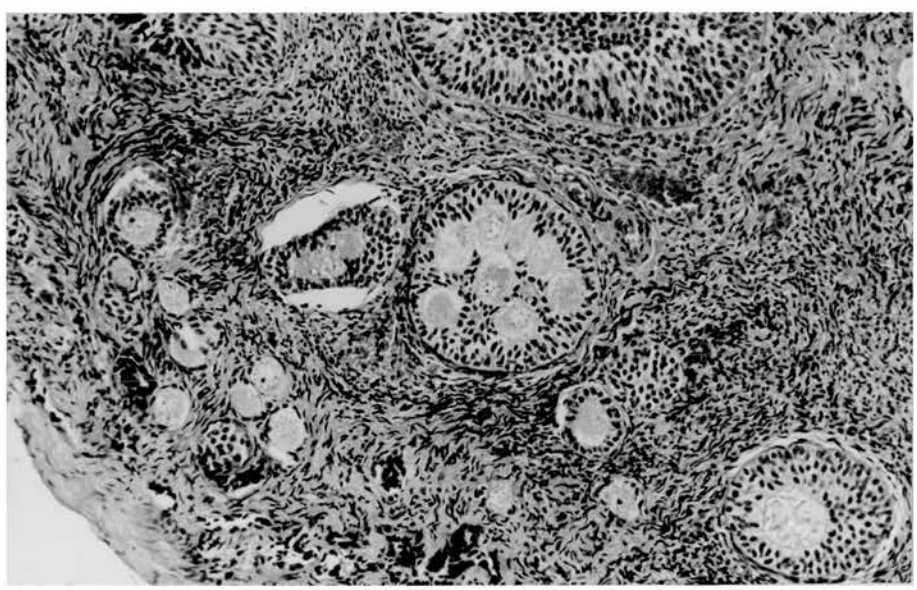


Figure 7.2c:

Detail of the cortex of the bitch ovary showing a growing follicle containing 5 oocytes. H & E. X320.

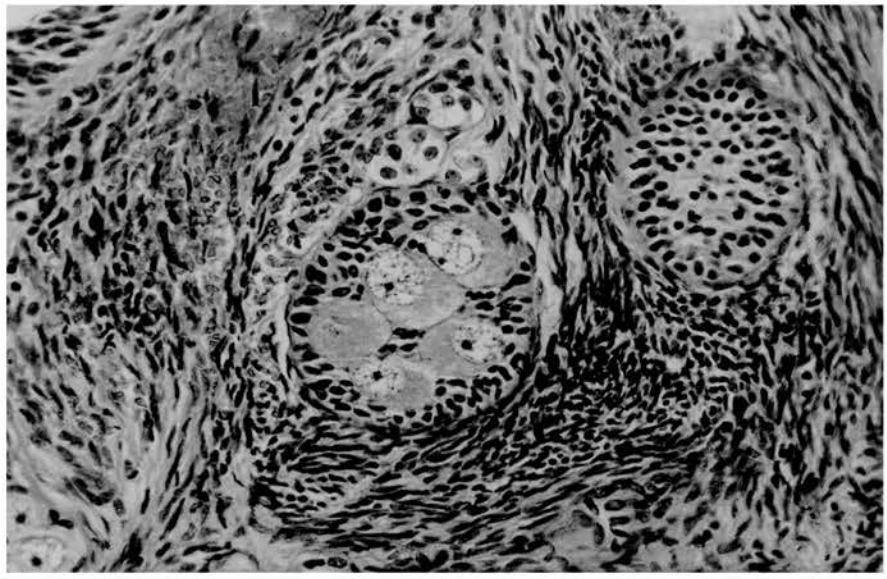


Table 7.4:
Mean contribution of nongrowing and growing follicles in young and old animals; contribution of polyovular follicles (pof) to growing population of follicles.

	Young animals	Old animals
% of nongrowing follicle	90	49
% of growing follicles	10	51
% of pof in growing follicles	14	5

Table 7.5:
Correction factors applied to obtain estimate of follicle numbers per ovary.

Type / Stage	1	2	3	4	>4
II	14.80	***10.83***	***9.30***	***9.0***	***9.0***
III	13.80	10.83	9.30	9.0	9.0
IV	13.40	***10.83***	***9.30***	***9.0***	***9.0***
V	13.20	***10.83***	***9.30***	***9.0***	***9.0***

Correction factor for stage I (small non growing follicles) = 15

Values in bold italics have not been estimated but are the values obtained for stage III polyovular follicles and were used to estimate the total numbers of polyovular follicles at other stages of development.

In the older group of animals anovular structures were observed, i.e. follicles in which the oocyte had degenerated and was invaded by granulosa cells.

Correction factors:

The data obtained from the follicle counts are arranged as proportions and are shown in figures 7.3 and 7.4 represented as histograms to show the mean proportion of each type of follicle at the four stages of follicular development. These proportions are based on data obtained from counting every 20th section, multiplied by the sampling frequency and a correction factor applied. To obtain an estimate of total follicle numbers some workers have multiplied the value obtained by the sampling frequency used. This method can lead to inaccuracies and overestimation of the total numbers. Correction factors to account for over estimation and based on the use of a discrete marker were formulated to obtain better estimates of total counts. In this study the correction factor of Abercrombie (1946) was used and this can be applied to follicles with only one oocyte at all stages of development. Problems arise when trying to obtain a correction factor for those follicles containing more than one oocyte since there is more than one marker, thus, strictly speaking the Abercrombie correction factor cannot be applied. In these cases an empirical correction factor was obtained for stage III follicles with more than one oocyte. Table 7.5 gives the values of the correction factors for each stage of follicle development. Table 7.10 shows nucleolar diameters from which some of the correction factors were obtained.

Figure 7.3:

Percentage of follicles with 1,2,3,4 & 5+ oocytes at each successive stage of follicular development in ovaries of young bitches. Values given are mean (+S.E.M) N=10.

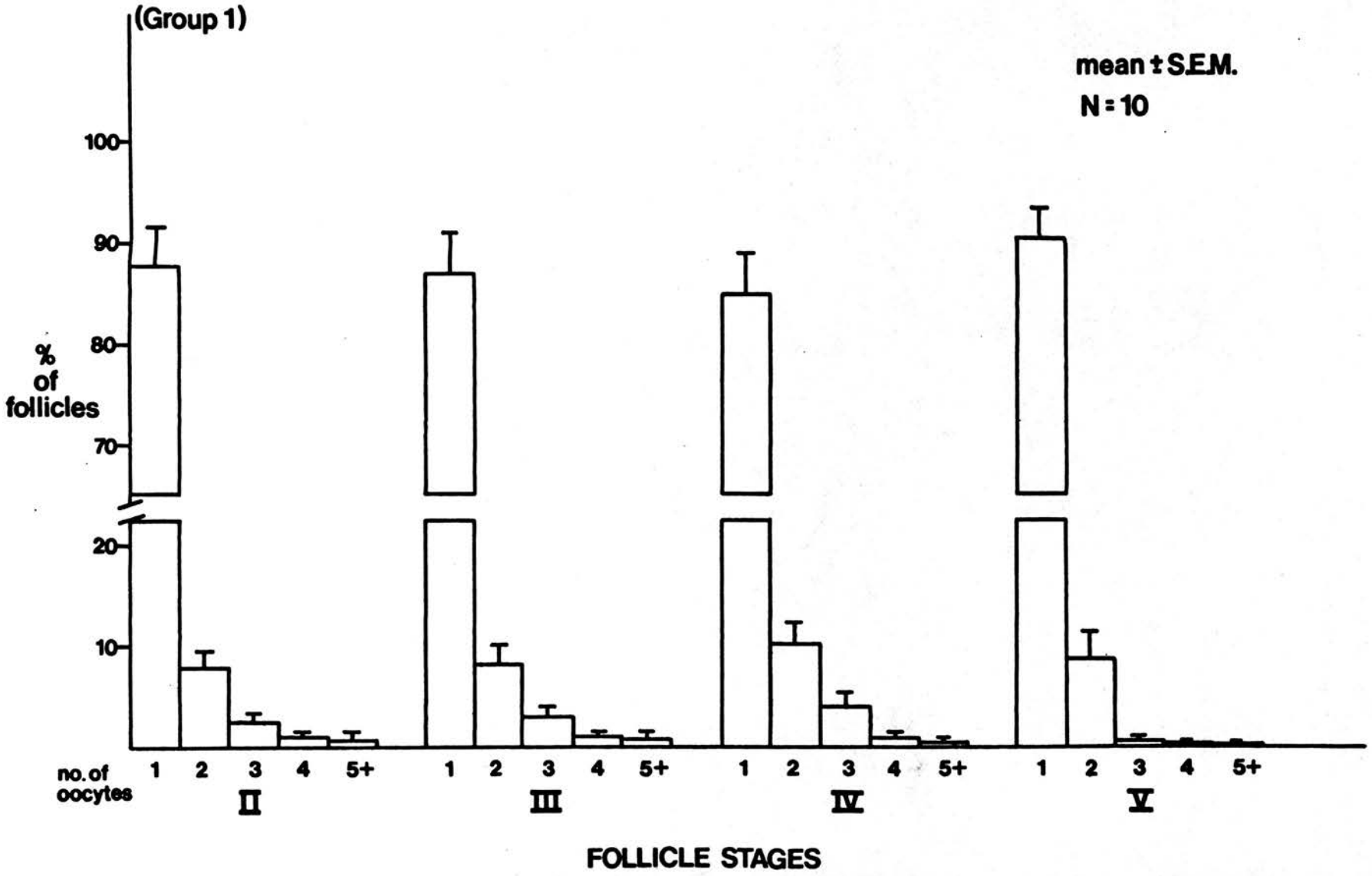
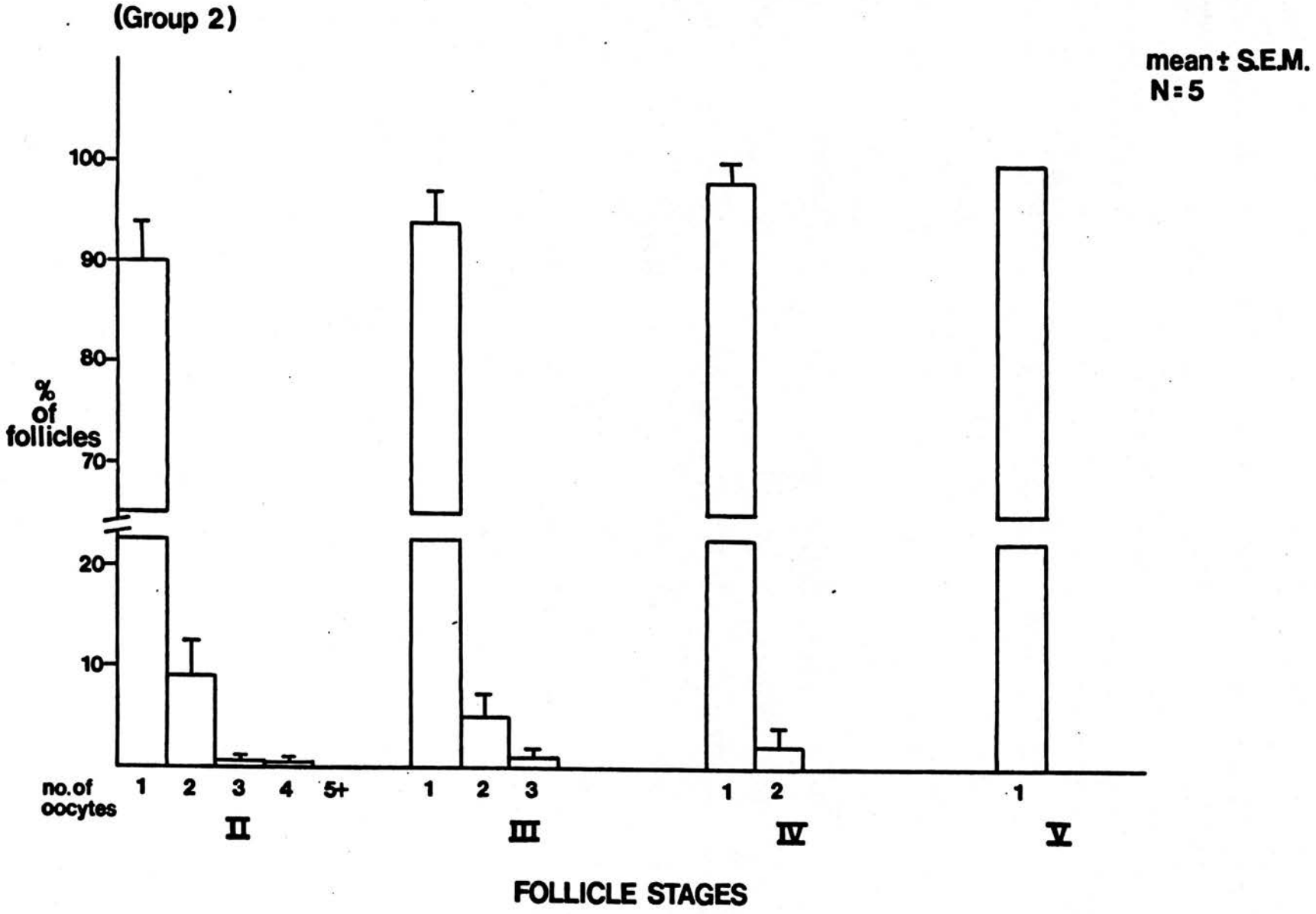

(Group 1)
mean ± S.E.M.
N = 10
100
90
80
70
20
10
% of follicles
no. of oocytes
1 2 3 4 5+
II
III
IV
V
FOLLICLE STAGES

Figure 7.4:

Percentage of follicles with 1,2,3,4, & 5+ oocytes at each successive stage of follicular development in the ovaries of ageing bitches. Values given are mean (+S.E.M.) N=5.


(Group 2)
mean ± S.E.M.
N = 5
100
90
80
70
20
10
% of follicles
no.of oocytes
1 2 3 4 5+
1 2 3
1 2
1
II
III
IV
V
FOLLICLE STAGES

Estimation of total follicle numbers:

Tables 7.6 and 7.7 give the estimated values of total follicle numbers at each stage of development and according to numbers of oocytes in a pair of ovaries for both young and old groups. The estimated total numbers of polyovular type follicles at stages II, IV, & V were estimated on the basis of the empirical correction factor obtained for polyovular follicles types at stage III as it would be laborious to estimate correction factors for polyovular follicles at each stage of development (these values are indicated in bold in tables 7.6 & 7.7). These data are not presented as definitive figures but rather to give an indication of numbers involved and indeed the values obtained are in accordance with other estimates of follicle numbers in the bitch.

Standard deviations were high showing that total numbers varied considerably between animals. There was a clear pattern of declining numbers with stage of development and with follicle types within each stage. Comparisons between the young and old group of animals shows a decline in the numbers of follicles with age, stage I follicles (non-growing follicles) decline from a mean of 85782 in young animals to a mean of 2754 in the older group. Numbers of follicles in the growing pool are also reduced in the older animals.

Uncorrected data versus corrected data:

For the purpose of analysis it was decided to treat the data on follicle counts as proportions it was neccesary to determine whether the results would be affected by the use of the raw or corrected data. Since different correction factors have been used.

Table 7.6:

Estimated mean total follicle numbers for a pair of ovaries in young bitches.

Type / Stage	1	2	3	4	>4
II	4002	**378**	**119**	**56**	**49**
	(1850)	*(328)*	*(147)*	*(109)*	*(107)*
III	2089	210	90	40	27
	(101)	*(244)*	*(135)*	*(180)*	*(63)*
IV	972	**147**	**59**	**20**	**5**
	(418)	*(191)*	*(95)*	*(41)*	*(17)*
V	*1251*	*128*	*13*	*4*	*2*
	(660)	*(190)*	*(45)*	*(11)*	*(6)*

Estimated mean total number of stage I follicles and standard deviation for ten young bitches = 85782 (+/- 58918).

Values in bold have been estimated using the correction factor calculated for stage III polyovular follicles (see Table 7.5).

Table 7.7:

Estimated total mean numbers of follicles for a pair of ovaries in old bitches

Type / Stage	1	2	3	4	>4
II	1308	**100**	**8**	**4**	0
	(866)	(85)	(10)	(8)	
III	828	30	4	0	0
	(604)	*(33)*	*(8)*		
IV	343	**4**	0	0	0
	(389)	*(9)*			
V	364	0	0	0	0
	(374)				

Estimated mean number of stage I follicles and standard deviation from five old bitches = 2754 (+/- 2021).

Values in bold have been estimated using the correction factor calculated for stage III polyovular follicles (see Table 7.5).

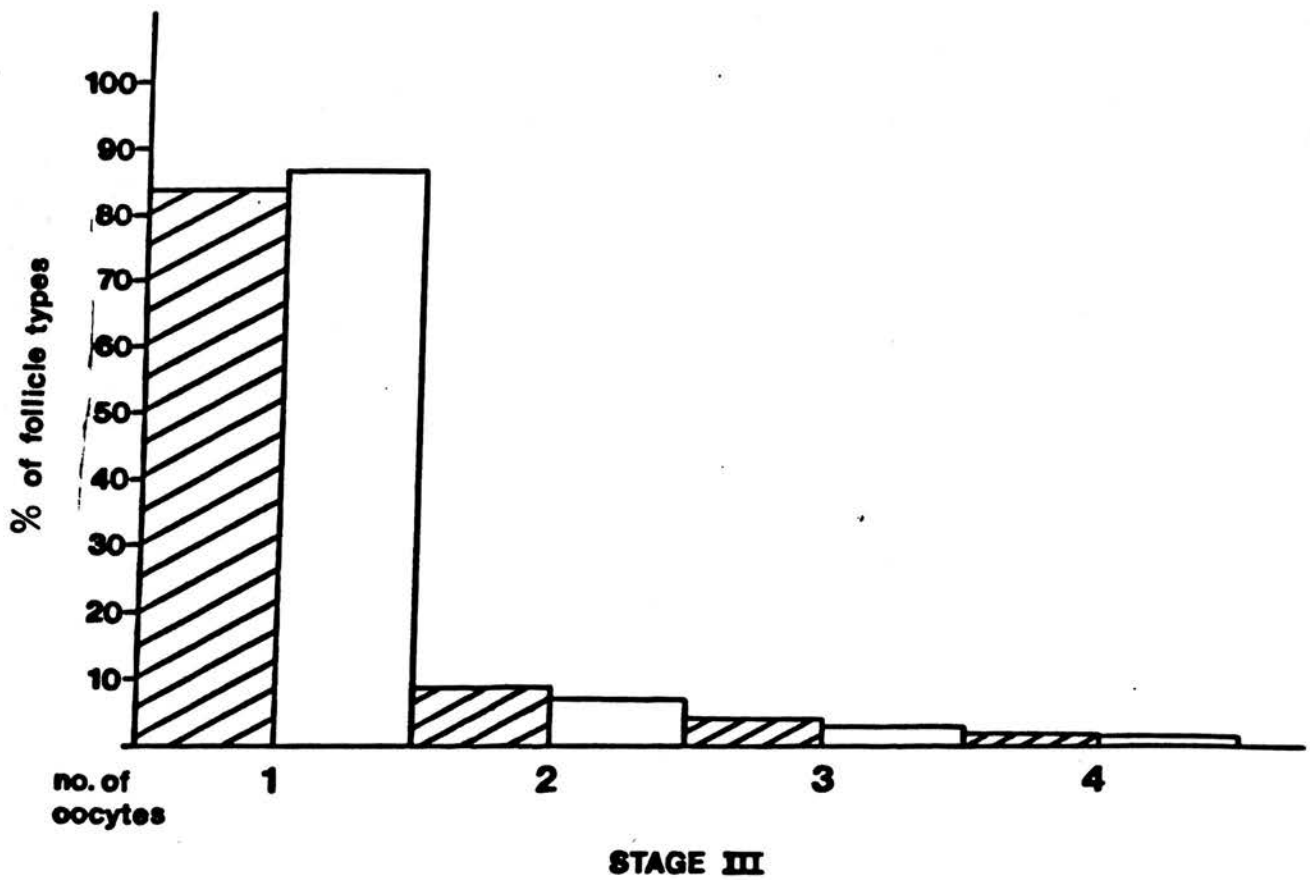
The main criticism of such an approach could be that there would be differences in probability of counting certain follicle types and stages and the correction factor should allow for these. Data corrected using the obtained correction factors were transformed into proportions and compared with uncorrected data. Figure 7.5 shows the results and there were no significant differences between corrected and uncorrected data. Thus, data presented have been corrected.

Group 1:

The total number of follicles counted varied considerably from animal to animal with those at stage I ranging from 746 - 7124 with a mean of 2859. Estimated total numbers per pair of ovaries ranged from 22388 - 213720 with a mean of 85782 (Table 7.6). Follicles containing more than one oocyte were found at all stages of follicular development in nine of the ten younger animals. These polyovular follicles accounted for a mean of 14% of the growing population of follicles (see Table 7.3). When polyovular follicles were subdivided into those containing 2, 3, 4 and 5^{+} oocytes the proportions of each type were remarkably consistent from stage to stage and followed a pattern of descending order at any given stage (Figure 7.3). The results of a non-parametric analysis of variance test (Kruskal- Wallis test) suggested no significant difference in the pattern of distribution of follicle types at any given stage in the young animals. There was a reduction in numbers of follicles from stage to stage but the proportion of polyovular follicles at a given stage of development up to stage V of development, where there was a decline in the proportion of type 4 and type 5^{+} follicles observed, was remarkably similar from stage to stage and between animals.

Figure 7.5:

Mean percentage of stage III follicles containing 1, 2, 3 & 4 oocytes in young bitches. Hatched bars represent data obtained from raw (uncorrected) counts of follicles and plain bars represent data obtained from counts after a correction factor has been applied.



Group 2:

In the older group of animals where the ages ranged from 7-11 years old, actual counts of stage I (primordial) follicles ranged from 40-205 with a mean of 92, estimated numbers per pair of ovaries ranged from 200 - 6150 with a mean of 2754 (Table 7.7). Polyovular follicles were found in all of the five older animals; however, they were not found at all stages of development, no polyovular follicles at stage V of development were found. Polyovular follicles accounted for a mean of 5% of the growing population of follicles in this older group (Table 7.3), this being significantly less than the percentage of polyovular follicles found in the young group. At stages II and III polyovular follicles followed a pattern of descending order according to number of oocytes similar to that in the younger group (Figure 7.4), but type 5^{+} polyovular follicles were not found in the older group of animals.

Probabilities

Tables 7.8 and 7.9 show the simple probabilities, calculated from the total counts, for each type of follicle in the growing pool of follicles, i.e. all follicle types ($1-5^{+}$) at stages II-V. The probability of observing a stage II type 1 follicle is greater in the older group than in the younger group and the probability of observing polyovular follicles of any stage decreases in the older group. The probability decreases with number of oocytes with the probability of finding a stage V follicle with greater than four oocytes enclosed is less than .001, thus if a low sampling frequency was chosen these types may not be observed.

Table 7.8:
Simple probabilities for each follicle type and stage in the growing population of follicles in young bitches.

Stage \ Type	1	2	3	4	>4
II	0.414	0.040	0.013	0.006	0.005
III	0.216	0.022	0.009	0.004	0.003
IV	0.100	0.015	0.006	0.002	<0.001
V	0.129	0.013	0.001	<0.001	<0.001

Table 7.9:
Simple probabilities of follicle types in growing population in older bitches.

Stage \ Type	1	2	3	4	>4
II	0.440	0.033	0.003	0.001	<0.001
III	0.276	0.010	0.001	<0.001	<0.001
IV	0.114	0.001	<0.001	<0.001	<0.001
V	0.122	<0.001	<0.001	<0.001	<0.001

Follicle Measurements:

Table 7.10 gives the values obtained from measurements of oocyte, nuclear, nucleolar and follicular diameters. From these data correlation coefficients (r) were obtained. Table 7.11 lists the correlation coefficients (r) obtained between several follicular variables throughout development and with varying number of oocytes. Significant correlations were obtained between all the variables measured in uniovular follicles. These results indicate that growth (as measured by increase in size) proceeds simultaneously in all variables. Low values were obtained for correlations of nucleolar diameter and the other variables when two and greater than five oocytes were present.

Individual oocyte development in polyovular follicles:

The data represented on figure 7.6 shows the diameter of individual oocytes from follicle stages II - V, types 1 - 5+. In follicles classified as stage II individual oocyte diameter is independent of the number of oocytes enclosed in the follicle. At stages III, IV, & V there is a decrease in the mean diameter of oocytes in polyovular follicles. Figure 7.7 a-e depicts the relationship between individual oocyte and follicle diameters for follicles containing 1, 2, 3, 4 and 5+ oocytes. Here the increase in diameter was used as the indicator of growth. Statistically significant correlation coefficients (r) were obtained for oocyte and follicle diameters in all follicle types. The r values obtained ranged from .885 for type 1 follicles to .713 for type 5+ follicles.

Table 7.10:
Mean (+ Standard deviation) diameter in microns (μ) of oocyte, nucleus, nucleolus and follicle in uniovular and polyovular follicles through successive stages of follicular development.
20 follicles of each stage & type were measured except >4 where 10 follicles were measured.

Stage	Variable	Follicle type 1	2	3	4	>4
I	oocyte	27.91 (3.88)				
	nucleolus	3.28 (.42)				
	nucleus	17.68 (3.27)				
	follicle	35.86 (5.10)				
II	oocyte	32.98 (4.64)	30.74 (3.90)	33. 77 (4.50)	30.50 (4.20)	30.80 (4.34)
	nucleolus	3.40 (0.63)	2.96 (0.66)	3.42 (0.42)	3.38 (0.45)	3.98 (0.43)
	nucleus	18.64 (2.99)	18.15 (2.24)	17.38 (2.29)	16.79 (4.23)	17.40 (3.01)
	follicle	46.77 (9.86)	69.98 (6.53)	88.30 (8.55)	88.90 (9.5)	120.30 (28.60)
III	oocyte	59.73 (13.50)	38.77 (5.70)	39.99 (6.75)	41.80 (4.78)	44.35 (13.33)
	nucleolus	4.34 (0.56)	3.53 (0.37)	3.76 (0.47)	3.96 (0.43)	3.81 (0.65)
	nucleus	23.30 (2.58)	19.33 (4.29)	19.25 (3.45)	21.30 (3.95)	22.14 (5.32)
	follicle	108.60 (23.3)	95.60 (22.08)	137.40 (19.77)	142.91 (9.54)	158.00 (28.86)
IV	oocyte	93.74 (10.60)	68.20 (11.11)	60.78 (20.40)	67.96 (16.04)	50.00 (10.00)
	nucleolus	4.87 (0.39)	4.07 (0.53)	4.19 (0.52)	4.21 (0.46)	4.17 (0.40)
	nucleus	27.30 (5.56)	25.46 (3.14)	22.59 (2.70)	25.17 (3.30)	20.10 (4.06)
	follicle	161.00 (30.40)	171.82 (32.80)	177.63 (37.90)	237.60 (21.79)	219.00 (24.70)
V	oocyte	108.29 (17.16)	95.50 (6.66)	85.20 (28.30)	88.90 (25.73)	
	nucleolus	4.93 (0.62)	5.31 (0.87)	4.92 (0.83)	4.76 (0.67)	
	nucleus	33.70 (6.29)	28.26 (4.87)	26.20 (6.30)	29.20 (4.12)	
	follicle	279.00 (89.80)	343.00 (101.00)	391.00 (72.40)	387.00 (65.80)	

Table 7.11:
The Correlation coefficients (r) of oocyte, nucleolar, follicle and nuclei diameters in 5 types of follicles throughout follicular development.

Follicle Type		1	2	3	4	>4
Variables						
a	b					
oocyte	nucleolus	.765	.328	.628	.752	.259
oocyte	nucleus	.750	.748	.713	.735	.703
oocyte	follicle	.885	.876	.734	.858	.713
nucleolus	nucleus	.632	.426	.594	.676	.166
nucleolus	follicle	.667	.330	.689	.683	.176
nucleus	follicle	.702	.729	.639	.768	.509

Those values in bold represent r values not statistically significant at the 5% level.

Figure 7.6:

Variation in the diameters of oocytes in uni- and polyovular follicles at different stages of development in bitch ovaries. Values are mean + s.e.m. number of oocytes measured at each stage = 20 (type 1); 20 (type 2); 30 (type 3); 20 (type 4) & 40 (type 5+).

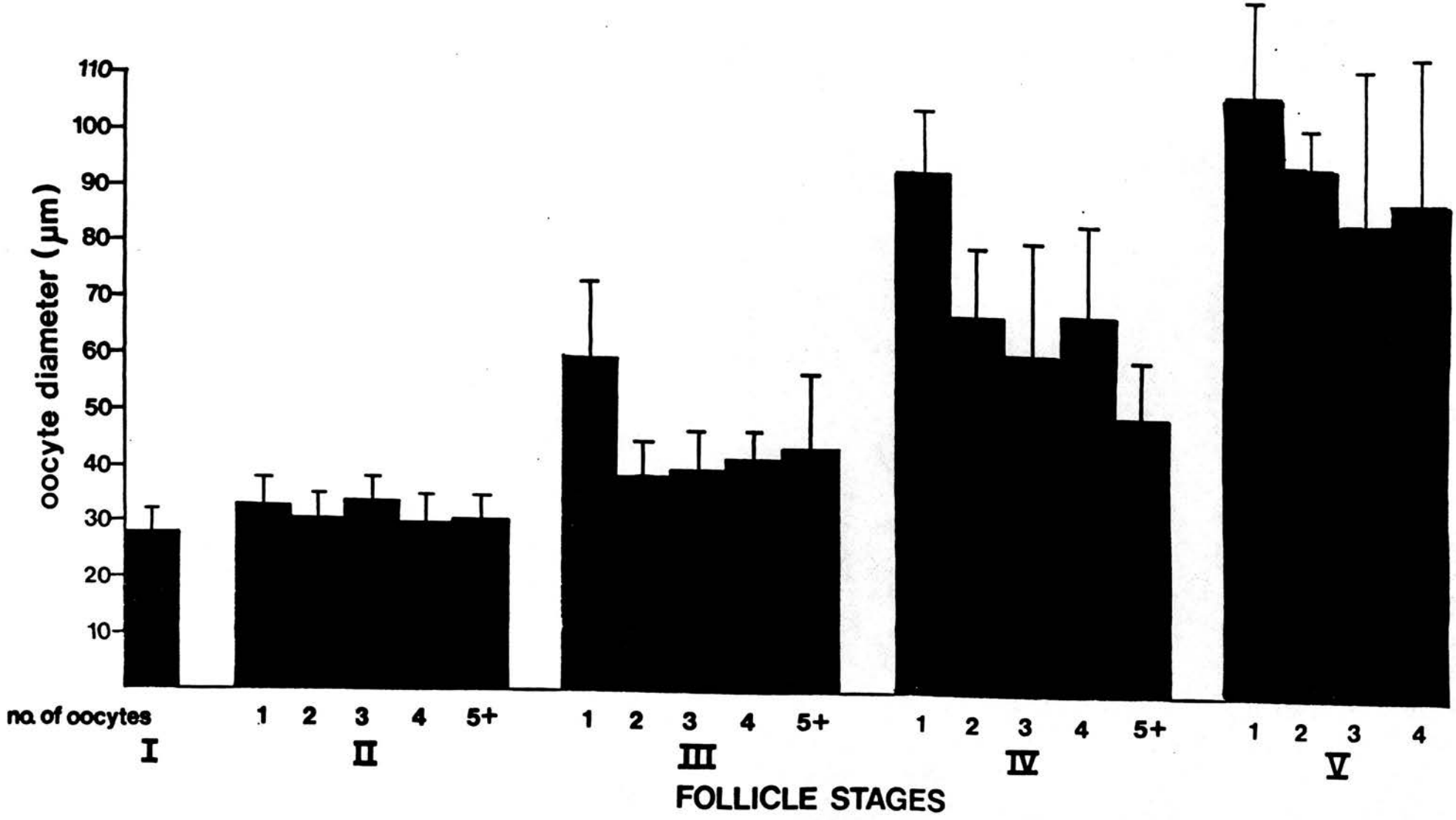
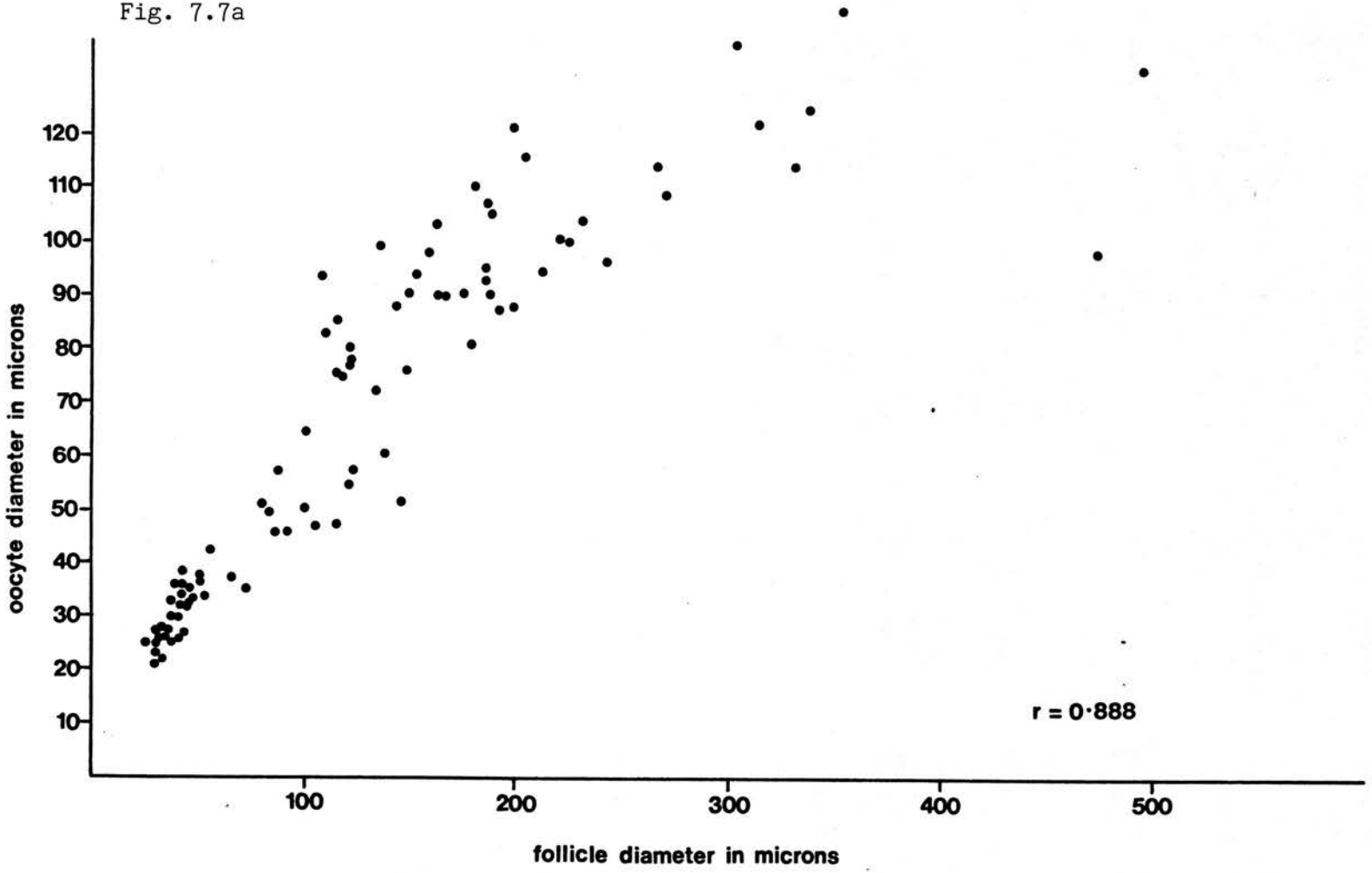

oocyte diameter (µm)
110
100
90
80
70
60
50
40
30
20
10
no. of oocytes
1 2 3 4 5+
1 2 3 4 5+
1 2 3 4 5+
1 2 3 4
I
II
III
IV
V
FOLLICLE STAGES

Figure 7.7a-d:

Figures 7.7 a-d are shown on pages 200-203. These graphs represent the relationship between follicle growth and oocyte growth in follicles containing one oocyte (7.7a); two oocytes (7.7b); three oocytes (7.7c) and four oocytes 7.7d. The correlation coefficient (r) is given on each graph.

1 oocyte per follicle

Fig. 7.7a



2 oocytes per follicle

Fig. 7.7b

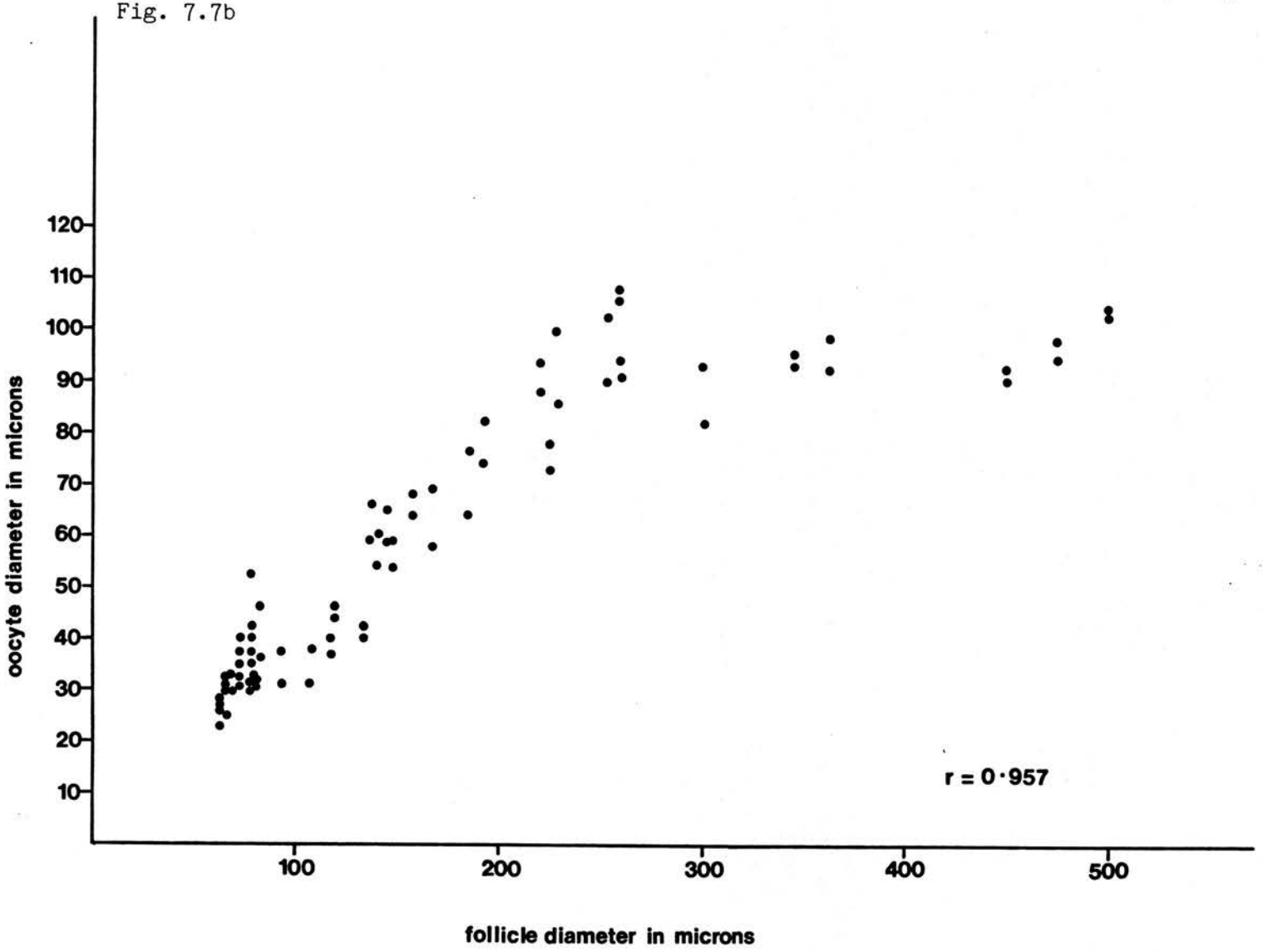
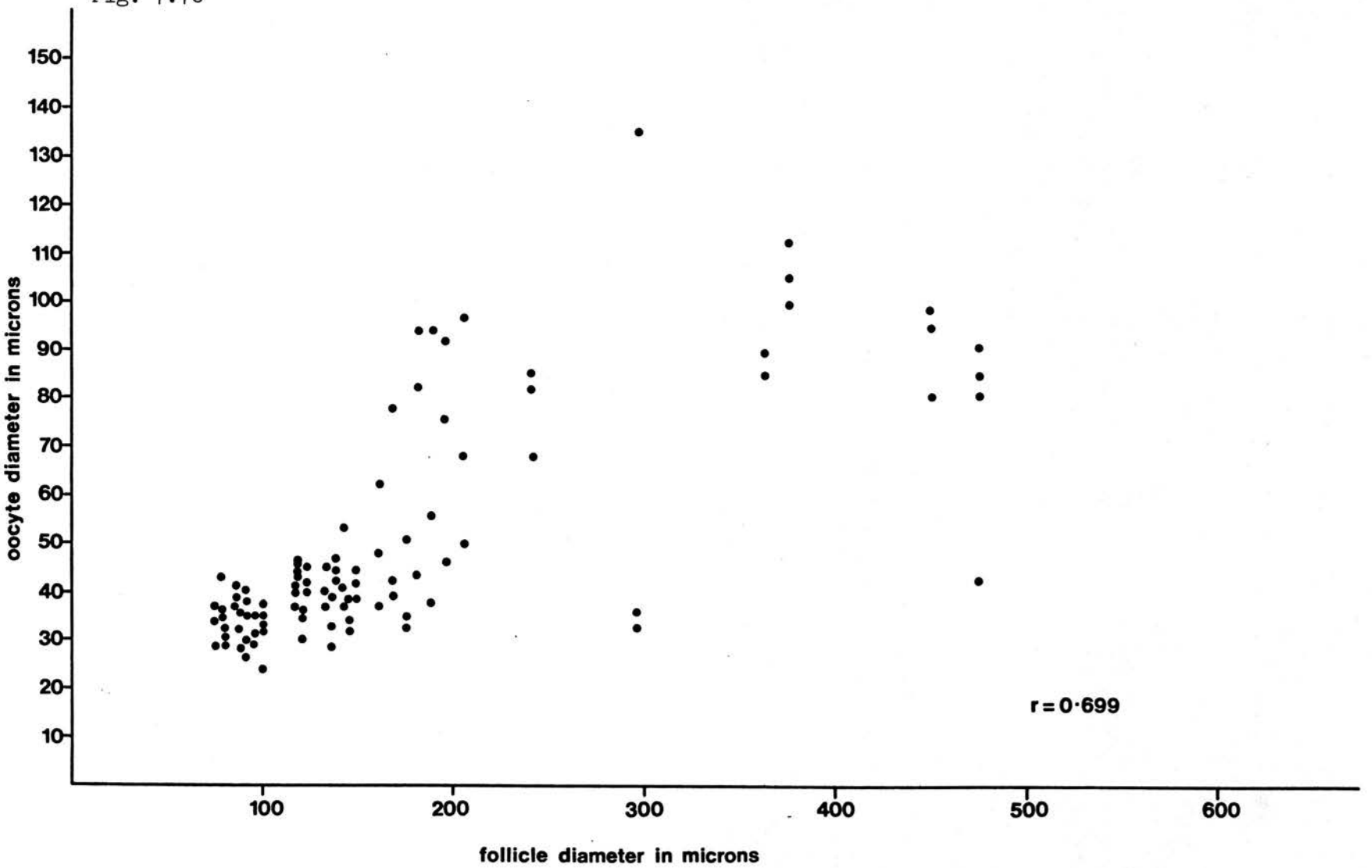


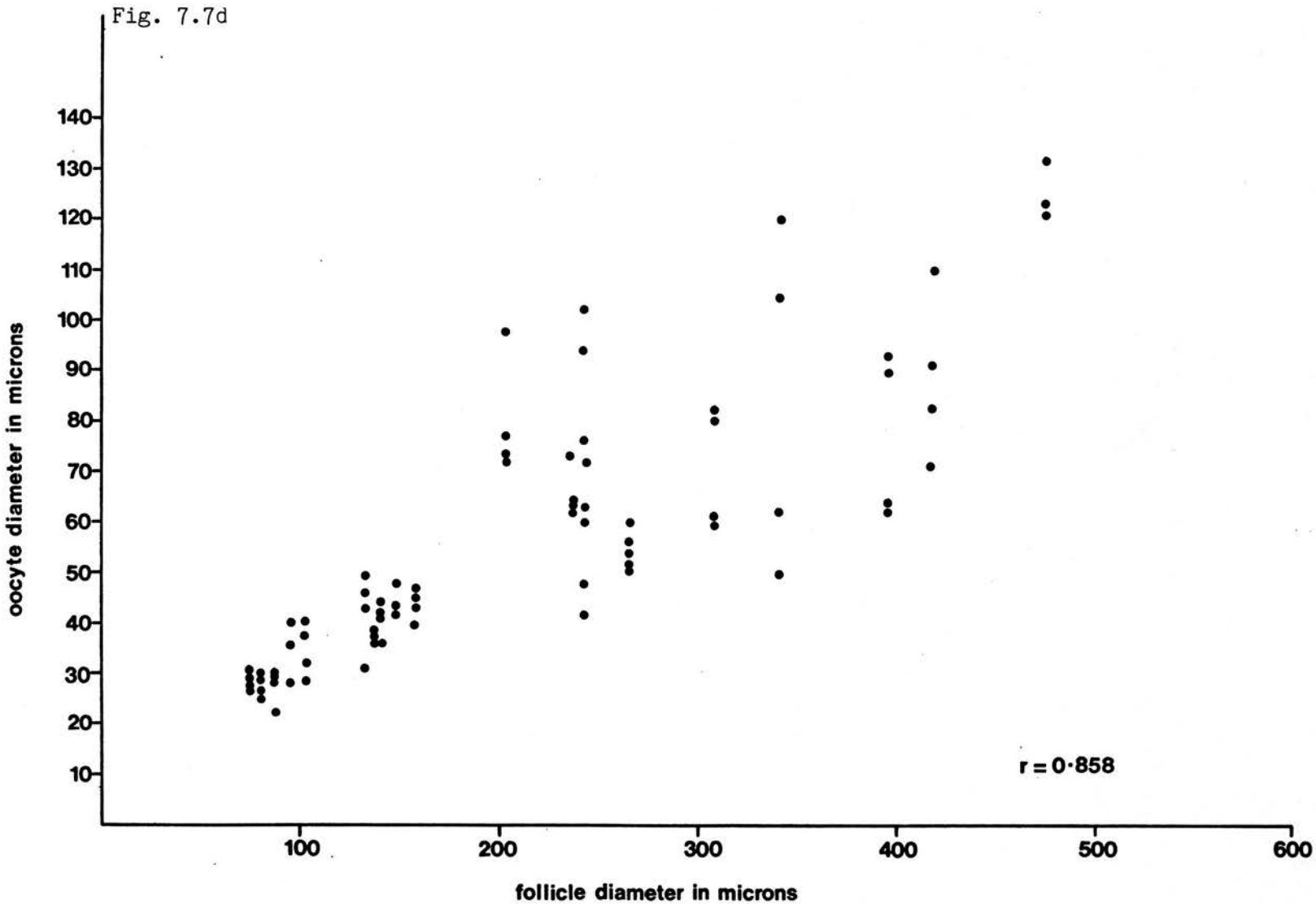
Fig. 7.7c

3 oocytes per follicle



4 oocytes per follicle

Fig. 7.7d



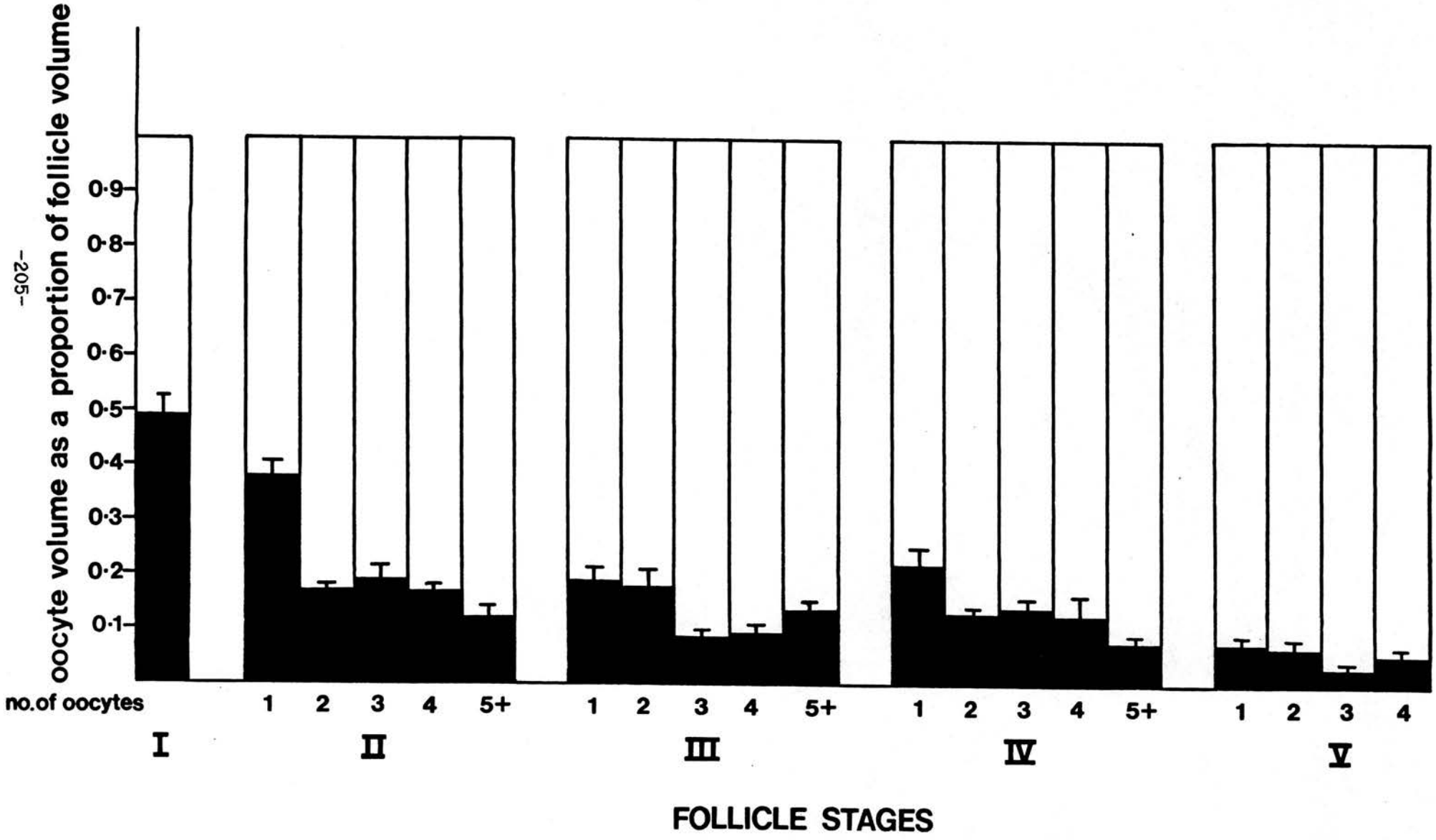
These data suggest a linear relationship exists between growth of follicle and individual oocyte independently of the number of oocytes within the follicle. On figure 7.7 graphs c & d show a greater scattering of points as follicular diameter increases. This is possibly an indication of a lesser degree of correlation between oocyte and follicle diameter as follicular development proceeds.

Oocyte vol - granulosa cell vol

Figure 7.8 represents the percentage volume of the follicle occupied by the oocytes *in toto* (shaded area) and so the light area may be considered to be mainly indicative of the volume occupied by the granulosa cells, extracellular volume being small in pre-antral follicles. The proportion of oocyte volume was significantly greater in uniovular stage II follicles than in polyovular follicles at this stage. Thus, it is assumed that proportionately more granulosa cells were associated with the poly ovular follicles. The ratio of oocyte volume to granulosa cell volume was not significantly different between those follicles with two, three, four and >four oocytes. At intermediate stages of development the proportions varied with follicles containing one oocyte and those with two oocytes at stage III being similar but with uniovular differing at stage IV. At stage V no differences between follicles with one or two oocytes were observed.

Figure 7.8:

This figure represents the proportion of follicle volume occupied by the oocyte(s) (shaded area) at each stage of development and in each type of follicle. Values are given as mean and standard deviation as standard errors were too small to be drawn.


oocyte volume as a proportion of follicle volume
0·9
0·8
0·7
0·6
0·5
0·4
0·3
0·2
0·1
no.of oocytes
1 2 3 4 5+ 1 2 3 4 5+ 1 2 3 4 5+ 1 2 3 4
I
II
III
IV
V
FOLLICLE STAGES

Discussion:

This study has shown that polyovular follicles in the bitch account for a significant proportion of the growing follicle population and the data suggest that polyovular follicles are a natural polymorphism rather than a spurious or pathological phenomenon. Uniovular development is the dominant mode in all species investigated; however, polyovuly is widely distributed but at varying frequencies.

Polyovuly poses a number of fundamental questions about the developmental biology of oocytes and follicles, namely: What are the predisposing factors leading to their formation, is their developmental potential impaired compared with that of uniovular follicles and do they contribute to fecundity? The subject of polyovular follicles has been tackled superficially in the past and many workers have built up hypotheses based on sparse data and inadequate materials. Further confusion arises because much of the work on polyovular follicles pre-dates our knowledge of the developmental biology of follicles and of modern endocrinology. This discussion will review the major hypotheses that have been presented and analyse these in relation to the present findings.

Hartman (1926) proposed three modes of origin of polyovular follicles:

1) The division of polynuclear ova
2) The concrescence of previously separated follicles.
3) The failure of ova to separate and to become surrounded by a common layer of granulosa cells.

The first hypothesis has recently been proposed as a mechanism for the formation of polyovular follicles in mice (Iguchi *et al.*, 1986) but this can be refuted in the present case since polynuclear oocytes were rare (<0.001), and if this were a significant mechanism a disproportionate number of polyovular follicles with an even number of oocytes enclosed would be expected; this was not observed. In any case, the presence of large numbers of polynuclear oocytes does not neccesarily imply that polyovular follicles will occur as some species have been found to have ovaries containing many polynuclear ova but no polyovular follicles (Hartman, 1926).

If the concrescence of previously separated follicles was a mechanism for formation of polyovular follicles in the bitch it should be possible to observe transition stages of the fusion process. In the present study no morphological evidence could be found to support this theory. The concresence theory was originally proposed by Loeb in 1917 based on the observations of primordial follicles closely associated with larger follicles.

The third hypothesis has been supported on the grounds that polyovular follicles are accidents of development due to a variable proportion of germ cells, epithelium and stroma, and that it is the crowding of an excessive number of germ cells in proportion to the presumptive granulosa cells that plays the largest part (Hartman, 1926; Evans & Swezy 1931). The data from this study are consistent with, but do not prove the hypothesis that polyovular follicles are predetermined during folliculogenesis. There is circumstantial evidence for the predetermination of polyovular follicles such as the clumps of oocytes

that appear to be surrounded by a single layer of granulosa cells but whether these constitute a single structure can not be validated at the light microscope level. Further cytological studies are required to determine the intimacy between such clusters of oocytes and whether metabolic coupling exists. It is interesting that cortical oocytes occur in dense plaques in young ovaries of species having abundant polyovular follicles, but density dependence cannot be the only causal factor since intimately clustered oocytes occur in species which rarely present polyovuly (e.g. sheep).

A major feature of the data is the inverse relationship between the number of oocytes per follicle and the number of follicles. The distribution of follicle types suggests that a stochastic process has been operating although this cannot be verified in the adult ovary since utilisation and death of follicles has altered the original population and, furthermore, incipient polyovular follicles cannot be identified at the light microscope level. The population of follicles will be fixed by about 54 days post-partum in the bitch with oogenesis not being completed until after birth (Andersen & Simpson, 1973). This differs from many other species in which oogenesis is complete before or shortly after birth (Beaumont and Mandl, 1962).

Folliculogenesis begins at around 17 days of age in the beagle (Andersen & Simpson 1973) and at 53-54 days of age the ovarian cortex is mainly composed of primordial follicles isolated or in clusters. The frequently observed clumps of oocytes surrounded by a single layer of squamous granulosa cells are probably the precursors of polyovular follicles which become clearly identifiable during follicular growth.

Thus, from the observations it is assumed that polyovular follicles are formed during folliculogenesis and that their population will be fixed at the end of this process and so they will make up a proportion of the primordial pool of follicles from which all growing follicles develop. This conclusion that polyovuly arises through random events conflicts with most suggestions in the older literature.

Several workers have proposed that gonadotrophic and steroid hormones influence the formation and development of polyovular follicles. Studies on rats (Kent 1962a), mice (Kent, 1960), guinea pig (Collins & Kent, 1964) and the golden hamster (Kent, 1958, 1962b, 1964; Kent & Mandel, 1968, 1970), looked at control and oestrogen treated groups and concluded that an inverse relationship between plasma oestrogen levels and the incidence of polyovular follicles existed, the implication being that oestrogen prevents the formation of polyovular follicles. In contrast with the findings of Kent & Mandel (1968, 1970) it has been reported that high levels of oestrogen are correlated with a high level of polyovular follicles in the squirrel monkey (Graham & Bradley, 1971). This study suggested that high oestrogen levels may induce the reaggregation of primary oocytes by migration and loss of the follicular epithelium normally surrounding them. The evidence supporting an effect of oestrogen on the formation of polyovular follicles is not convincing and these correlations do not necessarilly imply causation and no mechanism of action has been postulated.

A role for the gonadotrophins in the induction of polyovular follicles was invoked by Lloyd & Rubenstein (1941) after observations of the ovaries of rhesus monkeys treated with gonadotrophins. After finding

abundant polyovular follicles they suggested that the gonadotrophins had activated oocytes in uniovular follicles to divide. The predictions of this hypothesis appear to oppose those of the oestrogen hypothesis, but the chief difficulties are theoretical since this work predated modern knowledge of endocrinology and developmental biology of the oocyte. It is unlikely that a dictyate oocyte would divide. Had these workers carefully designed their study and observed the ovaries of control rhesus monkeys they would have found that polyovular follicles were abundant in the ovaries of control rhesus monkeys (Mossman & Duke 1973). Bodemer & Warnick (1961a,b) found that injections of gonadotrophins did not affect the size of the follicular population in toto but significantly increased the number of both uni and polyovular follicles with antra thus indicating that polyovular follicles were capable of responding to gonadotrophins in a similar way as do uniovular follicles.

The attraction of linking the occurrence of polyovular follicles with varying hormone levels lies in the need to explain the high incidence of these structures in prepubertal animals which has been repeatedly pointed out in the past on the basis of non quantitative data, (Nunes, 1926; Davis & Hall, 1950). These earlier studies often presented data based on the observation of polyovular follicles, which were assumed to be physiologically different, during another study and as a result the "normal" follicles were often over-looked (Lloyd & Rubenstein, 1941). The important question is whether the total number of growing follicles are influenced by hormone treatment or whether polyovular follicles are affected differently; the work previously cited does not answer this problem and it is the lack of quantitative data and adequate controls

that are the major problems with such studies.

The present results do not deny a possible role for the gonadotrophins and sex steroids but any hormonal influence in these particular animals is difficult to assess since they were anoestrus. The predetermination hypothesis proposes an explanation for polyovuly on the basis of cellular interactions which may or may not depend upon gonadotrophins and sex steroids. Fluctuating hormonal levels are unlikely to provide a general mechanism for the formation of polyovular follicles because substantial individual and species differences exist.

Theories based on a hormonal cause for the incidence of polyovular follicles must assume that oocyte- follicle cell interactions affect the response of follicle cells to changing hormone levels. It is unclear whether the exponents of these theories (Kent & Mandel, 1968, 1970) are suggesting that oestrogens inhibit the formation of polyovular follicles or that they interfere with their ability to enter the growing phase. Little is known about factors affecting the initiation of follicle growth, but it is assumed to be a spontaneous process independent of hormonal influences. This event may occur independently in oocytes and the influence transmitted to physiologically coupled oocytes. Thus it may be expected that the probability of utilisation will vary with the number of oocytes within a follicle.

Studies on the follicular dynamics in mice (see chapter 2) have shown that there is a peak in numbers of follicles entering the growing phase in early life and also during the first 20 days of life there is a loss of more than 50% of the original stock of primordial follicles. If this

pattern is similar in other species then polyovular follicles would be more abundant in the immature phase as are most other types of follicles. The cause of these deaths are not fully understood but there have been suggestions that polyovular follicles are the first to succumb to the postnatal reduction in the young human ovary (Bacsich, 1949), although no physiological evidence has been provided.

In this study polyovular follicles were less common in older bitches than in animals beginning their reproductive life, they were fewer in number and comprised a lower percentage of the growing follicle population. This age effect could be due to a reduced frequency of predetermined polyovular follicles in the reserve pool as a result of increased utilisation at young ages and/or decreased viabilty of follicles entering their growth phase. In order to test the hypothesis that polyovular follicles become progressively more sparse throughout life, more specimens are required at appropriate ages. It has been suggested that polyovular follicles are used more rapidly (Lane, 1938) and more susceptible to atresia than uniovular follicles, hence their peak in immature ovaries (Zybina, 1980). In this study atretic uniovular and polyovular follicles were noted but not included in the final counts. There were no apparent differences in the incidence of atresia between uni and polyovular follicles at any given age but, in the older animals the incidence of atresia was greater in all follicle types.

Whilst polyovular follicles were not found at later stages of follicular development in older bitches, at earlier stages they were present in similar proportions as in the younger animals. Since the

animals were anoestrous it is unclear whether there is a hormonal basis for these findings. The diminishing frequency of polyovular follicles at each successive stage of growth suggests that they are less viable in older ovaries. The mechanism by which these follicles become less viable is unclear. The environment of the ageing ovary may be less capable of providing physiological support for the growth of follicles and this is consistent with the observed higher incidence of follicle death. The reduction in larger polyovular types in the older ovaries may be as a result of their low frequency and therefore the probability of finding these follicle types would reduce as the number of follicles diminished with ageing.

The probability of finding polyovular follicles would be a product of their incidence in the primordial pool and of the number of follicles entering the growing phase. Both the number of follicles in the primordial pool and the number of follicles starting to grow are greatest during immature ages, thus the probability of finding polyovular follicles must also be higher at this time. Confusion may have arisen in the past because of the way in which polyovular follicles were recorded. Single sections were studied and absolute numbers of polyovular follicles were recorded without noting the numbers of uniovular follicles, thus giving a false impression. Sampling frequency is also crucial especially in a species such as the domestic dog where a single ovary can give rise to more than 1000 histological sections. If a particular type of follicle is not abundant then they may go undetected if the sampling frequency is low.

Apart from the formation of polyovular follicles the developmental potential of these structures and of the oocytes within them are of interest. Most oocytes in polyovular follicles undergo morphologically normal development although they are smaller than those in uniovular follicles at the same stage of development. These size differences do not emerge until stage III when two granulosa cell layers are present. These results can suggest several things :

(1) Oocytes within polyovular follicles develop more slowly than corresponding oocytes in a uniovular follicle.

(2) There is heterogeneity in growth of oocytes in polyovular follicles.

(3) The classification of stages for uniovular follicles is not appropriate for polyovular follicles.

From the observations made in this study it would appear that when more than one oocyte is present within a follicle, growth rate (as determined by size of oocyte) will be similar in all oocytes up to a given stage of follicular development. There is evidence from work on the rabbit (Al Mufti *et al.*, 1987) that the position of an oocyte within the follicle may determine its future growth and developmental fate. Observations in this study on follicles containing more than three oocytes would support the findings of Al Mufti *et al.*, (1987) at later stages of follicular development in those follicles containing more than three oocytes the peripheral oocytes tend to be smaller, however there is no significant difference in sizes of binovular follicles and the situation for trinovular follicles is variable. The results of the morphometric analysis of the follicles suggests that where more than two oocytes are present the growth rate will be similar in all oocytes

up to a certain stage of development, after which the position of the oocyte within the follicle may determine its future growth.

There are clearly factors other than oocyte position within a follicle which determine their development. Large stage V follicles with 2/3 oocytes are found in which all oocytes appear normal and are of a comparable size. In this study binovular and trinovular Graafian follicles have been observed in cats and rodents in which each oocyte has a cumulus mass developing around it and this supports observations by other workers (Bodemer & Warnick, 1961a,b). No reports have been found where this occurs in follicles with greater than three oocytes, thus, spatial factors may have an effect. Heterogeneity of oocytes at later stages of development may also have arisen by developmental lagging of oocytes. If timing of oocyte development is due to intrinsic factors (Edwards, 1970) then all oocytes within the same follicle may not be " switched on" at the same time. The data obtained in this study would not support the view of developmental lagging as differences appear at later stages rather than from the outset, although there is always the possibility that lagging is occurring from an intermediate rather than the starting point of development. Polyovular follicles were larger than uniovular types at the same stage and it will be interesting to determine whether these differences continue into antral stages.

In addition to the problem of their development polyovular follicles have attracted interest because of their potential contribution to the quota of oocytes shed at ovulation. According to the results of this study their contribution is likely to be small, even in species having

abundant polyovular follicles, and virtually nil in older animals. The quantitative significance of these follicles can be illustrated by a simple example. The frequency of polyovular follicles ovulating would be a product of their abundance at antral stages and the number of ovulations. The data from this study indicates that the probability of a binovular follicle ovulating is low. The stage V group is heterogeneous and only a proportion of it will contribute to the pool from which selection for ovulation will be made. However, the data suggests that polyovular follicles will be present in similar proportions at later stages of development to those at earlier stages. There is no evidence to suggest that polyovular follicles are more susceptible to atresia than uniovular follicles at later stages of development in young animals, so we can assume that the pool from which ovulatory follicles would be selected contains polyovular follicles.

Even if polyovular follicles ovulated this may not always result in an increased litter size. Since if developmental lagging has occurred all oocytes may not be capable of being fertilised (Ota, 1934; Gougeon 1981,) or indeed all oocytes within a follicle may not be ovulated as there is some evidence of oocytes being retained within the follicle at ovulation and becoming atretic (Engle 1927).

This study of polyovular follicles in the bitch leads to the conclusion that these structures are not abnormal physiologically as has previously been suggested, but represent morphological variants which are the result of developmental interactions occurring during folliculogenesis. These follicles enter the growing pool and develop

normally up to antral stages. There is no evidence to suggest that polyovular follicles are incapable of being ovulated, however, selection of a polyovular follicle for ovulation does not necessarilly result in the full complement of ova being shed. It is unlikely that polyovular follicles result in increased fecundity of the individual, as their relative numbers and differences in developmental potential of oocytes within individual follicles, excludes them from being a significantly increased source of viable oocytes.

Abercrombie, M. (1946). Estimation of nuclear population from microtome sections. *Anat. Rec.* **94**, 239-247

Al Mufti, W., Bomsel-Helmreich, O., and Christides, J.Ph. (1987). Influence of topographic situation and number of oocytes on their development in polyovular follicles of the rabbit. *J. Reprod. Fert.* (In Press).

Alden, R.H. (1942).Periovarial sac in albino rat. *Anat Rec.* **83**, 421-435.

Allen, E. (1923). Ovogenesis during sexual maturity. *Am. J. Anat.* **31**, 439-481.

Allen, E. and Creadick, R.N. (1937). Ovogenesis during sexual maturity. The first stage, mitosis in the germinal epithelium as shown by the colchicine technique. *Anat. Rec.* **69**, 191-195.

Allen, E., Pratt, J.P., Newell, Q.U. and Bland, L.L. (1930).
Human ova from large follicles: including a search for maturation divisions and observations on atresia.
Am. J. Anat. **46**, 1-54.

Allen, W.M. and Corner, G.W. (1930). Physiology of corpus luteum; maintenance of pregnancy in rabbit after very early castration by corpus luteum extracts. *Proc. Soc. Exp. Biol. Med.* **27**, 403-405.

Altman, P.L., and Dittmer, D.S. (Ed). (1972). *Biology data book.* **1**. 2nd edn. Bethesda, MD: Fed. Am. Socs. Exp. Biol.

Anand Kumar, T.C. (1968). Oogenesis in the lorises. *Loris tardigradus lydekkerianus* and *Nycticebus coucang*. *Proc. R. Soc. Lond. B. Biol. Sci.* **169**, 167-176.

Andersen, A.C., and Simpson, M.E. (1973). *The Ovary and the Reproductive Cycle of the Dog (Beagle).* Geron-X Inc., Los Altos, California.

Ansell, J.D., and Micklem, H.S. (1986). Genetic markers for following cell populations. In. *Handbook of Experimental Immunology.*, (ed). D.M. Weir. Vol. 2. chapter, 56.

Arai, H. (1920), On the cause of hypertrophy of the surviving ovary after semi spaying (albino rat) and on the number of ova in it. *Am. J. Anat.* **28**, 59-79.

Arendsen de Wolff-Exalto, E. (1982). Influence of gonadotrophins in early follicle cell development and early oocyte growth in the immature rat. *J. Reprod. Fert.* **66**, 537-542.

Arnold, L. (1912). Adult human ovaries with follicles containing several oocytes. *Anat.Rec.* **6**, 413-422.

Arrau, J., Roblero, L., Cury, M. and Gonzalez, R. (1983). Effect of exogenous sex steroids upon the number of germ cells and growth of foetal ovaries grafted under the kidney capsule of adult ovariectomised hamsters. *J. Emb. Exp. Morph.* **78**, 33-42.

Atkins, G.L. (1969). *Multicompartmental Models for Biological Systems.* Methuen, London.

Austad, R., Lunde, A., and Sjaastad, O.V. (1976). Peripheral plasma levels of oestradiol-17β and progesterone in the bitch during the oestrous cycle, in normal pregnancy and after dexamethasone treatment. *J. Reprod. Fertil.* **46**, 129-136.

Austin, C.R. (1976). Specialization of gametes. In *Reproduction in mammals. Book 6. The evolution of reproduction:* 149-182. Austin, C.R. & Short, R.V. (Eds). Cambridge. Cambridge University Press.

Bacsich, P. (1949). Multinuclear ova and multiovular follicles in the young human ovary and their possible endocrinological significance. *J. Endocrinol.* **6**, i-ii

Baird, D.T., Bäckström, T., McNeilly, A.S., Smith, S.K., and Wathen, C.G. (1984). Effect of enucleation of the corpus luteum at different stages

of the luteal phase of the human menstrual cycle on subsequent follicular development. *J. Reprod. Fert.* **70**, 615-624.

Baker, T.G. (1963). A quantitative and cytological study of germ cells in human ovaries. *Proc. R. Soc. London, Ser. B* **158**, 417-433.

Baker, T.G. (1971). Electron microscopy of the primary and secondary oocyte. *Adv. Biosci.* **5**, 7-23.

Baker, T.G. (1972). Oogenesis and ovarian development. *In*: *Reproductive Biology*. Balin, H. & Glasser,S.R. (Eds). pp. 398-437. Excerpta Med. Found., Amsterdam.

Baker, T.G., and Franchi, L.L. (1967a). The fine structure of oogonia and oocytes in human ovaries. *J.Cell Sci.* **2**, 213-224.

Baker, T.G., and Franchi, L.L. (1967b). The structure of the chromosomes in human primordial oocytes. *Chromosoma* **22**, 358-377.

Baker, T.G., and Franchi, L.L. (1967c). The fine structure of chromosomes in bovine primordial oocytes. *J. Reprod. Fert.* **14**, 511-513.

Baker, T.G. and Scrimgeour, J.B. (1980). Development of the gonad in normal and anencephalic human fetuses. *J. Reprod. Fert.* **60**, 193-199.

Baker, T.G., Beaumont, H.M., and Franchi, L.L. (1969). The uptake of tritiated uridine and phenylalanine by the ovaries of rats and monkeys. *J. Cell Sci.* **4**, 655-675.

Baker, T.G., Challoner, S., and Burgoyne, P.S. (1980). The number of oocytes and the rate of atresia in unilaterally ovariectomised mice up to 8 months after surgery. *J. Reprod. Fert.* **60**, 449-456.

Balfour, F.M. (1878). On the structure and development of the vertebrate ovary. *Q. J. Microsc. Sci.* **18**, 383-438.

Bartlett, M.S. (1949). Some evolutionary stochastic processes. *J.R. Statist. Soc. Ser.* B. **11**, 211-229.

Bast, J.D. & Greenwald, G.S. (1977). Acute & chronic elevations in serum levels of FSH after unilateral ovariectomy in the cyclic hamster. *Endocrinology.* **100**, 955-966.

Batra, S.K. and Miller, W.L. (1985). Progesterone decreases the responsiveness of ovine pituitary cultures to Luteinising Hormone-Releasing Hormone. *Endocrinology.* **117**, 1436-1440.

Beaumont, H.M. and Mandl, A.M. (1962). A quantitative and cytological study of oogonia and oocytes in the foetal and neonatal rat. *Proc. R. Soc. Lond. Ser, B. Biol. Sci.* **155**, 557-579.

Best, P.B. and Bannister, J.L. (1963). Functional polyovuly in the Sei Whale, *Balaenoptera borealis. Nature.* **199**, 89.

Biggers, J.D., Finn, C.A., and McLaren, A (1962). Long term reproductive performance of female mice. I. Effect of removing one ovary. *J. Reprod. Fert.* **3**, 303-312.

Block, E. (1952). Quantitative morphological investigations of the follicular system in women. *Acta. Anat.* **14**, 108-123.

Bocharov, W.S. (1966). Multiovular follicles in the ovaries of mammals. *Adv. Sov. Biol.* **62**, 139-147.

Bodemer, C.W. and Warnick, S. (1961a). Polyovular follicles in the immature hamster ovary. II. The effects of gonadotropic hormones on polyovular follicles. *Fert. Steril.* **12**, 353-364.

Bodemer, C.W. and Warnick, S. (1961b). Polyovular follicles in the immature hamster ovary. I. Polyovular follicles in the normal intact animal. *Fert. Steril.* **12**, 159-169.

Borum, K. (1966) Oogenesis in the mouse. A study of the origin of the mature ova. *Exp. Cell Res.* **45**, 39-47.

Brambell, F.W.R. (1956), "Ovarian changes". In: *Marshall's Physiology of Reproduction.* vol. 1, part 1. pp. 397-542. A.S. Parkes (ed.). Longmans, Green and Co., New York and Toronto.

Braw, R.H. and Tsafriri, A. (1980a). Follicles explanted from pentobarbitone treated rats provide a model for atresia. *J. Reprod. Fert.* **59**, 259-265.

Braw, R.H. & Tsafriri, A. (1980b), Effect of PMSG on follicular atresia in the immature rat ovary. *J. Reprod. Fert.* **59**, 267-272.

Brinkley, H.J. & Young, E.P. (1969), Effects of unilateral ovariectomy or unilateral destruction of ovarian components on the follicles and corpora lutea of the non pregnant pig. *Endocrinology*, **84**, 1250-1256.

Brody, S. (1945). *Bioenergetics and growth.* New York, Reinhold.

Browne, C.L., Wiley, H.S., and Dumont, J.N. (1979). Oocyte follicle cell gap junctions in Xenopus laevis and the effects of gonadotropin on their permeability. *Science*, **203**, 182-183.

Buffler, G., and Roser, S. (1974). New data concerning the role played by progesterone in the control of follicular growth in the rat. *Acta Endocrinology.* **75**, 569-578.

Bücher, T., Bender, W., Hofner, H. and Linke, I. (1980). Quantitative evaluation of electrophoretic allo- and isozyme patterns. *FEBS Lett.* **115**, 319-324.

Bullough, W.S. (1942). The method of growth of the follicle and corpus luteum in the mouse ovary. *J. Endocr.* **3**, 150-156.

Burton, D.I., Ansell, J.D., Gray, R.A., and Micklem, H.S. (1982). A stem cell for stem cells in murine haematopoiesis. *Nature.* **298**, 562-563.

Butcher, R.L. (1977) Changes in gonadotropins and steroids associated with unilateral ovariectomy of the rat. *Endocrinology.* **101**, 830-840.

Butcher, R.L., (1985), Effect of reduced ovarian tissue on cyclicity, basal hormone levels and follicular development in old rats. *Biology of reproduction.* **32**, 315-321.

Butcher, R.L., and Page, R.D. (1981). Role of the aging ovary in cessation of reproduction. In: *Dynamics of ovarian function.* Schwartz, N.B. & Hunzicker-Dunn, M. (Eds). Raven Press. New York. pp. 253-271.

Byskov, A.G. (1974a). Does the rete ovarii act as a trigger for the onset of meiosis? *Nature.* **252**, 396-397.

Byskov, A.G. (1974b). Cell kinetic studies of follicular atresia in the mouse ovary. *J. Reprod. Fert.* **37**, 277-285.

Byskov, A.G. (1975). The role of rete ovarii in meiosis and follicle formation in the cat, mink and ferret. *J. Reprod. Fert.* **45**, 210-219.

Byskov, A.G. (1978a) Regulation of initiation of meiosis in fetal gonads. *Int. J. Andrology,* suppl. **2**, 29-38.

Byskov, A.G. (1978b) The meiosis inducing interactions between germ cells and rete cells in the fetal mouse gonad. *Ann. Biol. Anim. Biochem. Biophys.* **18**, 327-334.

Byskov, A.G. (1979a). Regulation of meiosis in mammals. *Ann. Biol. Anin. Biochim. Biophys.* **19**, 1251-1261.

Byskov, A.G. (1979b). Atresia. In. *Ovarian follicular development and function.* Midgley, A.R. Jr., & Sadler, (eds). Raven press, New York, pp. 41-57.

Byskov, A.G. (1986). Differentiation of Mammalian Embryonic Gonad. *Physiological Reviews* **66**, 71-117.

Byskov, A.G. and Lintern-Moore, S. (1973). Follicle formation in the immature mouse ovary: the role of the rete ovarii. *J. Anat.* **116**, 207-217.

Byskov, A.G. and Rasmussen, G. (1973). Ultrastructural studies of the developing follicle. In: *The Development and Maturation of the ovary and its Function*, H.Peters (ed.), Amsterdam: Excerpta Med., pp. 55-62.

Byskov, A.G., N.E. Skakkebæk, N.E., Stafanger, G. and Peters, H. (1977). Influence of ovarian surface epithelium and rete ovarii on follicle formation. *J. Anat.* **123**, 77-86.

Cahill, L.P., Clarke, L.J., Cummins, J.T., Driancourt, M.A., Carson, R.S., and Findlay, J.K. (1984). An inhibitory action at the ovarian level of ovine follicular fluid on PMSG-induced folliculogenesis in hypophysectomized ewes. In: *Proceedings of the 5th Ovarian Workshop.* pp.35-38. Toft, D.O. & Ryan, R.J. (Eds). Ovarian workshops, Champaign, Illinois.

Calder III, W.A. (1984). *Size, function and life history.* Cambridge, MA: Harvard University Press.

Cattanach, B.M. (1975). Control of X chromosome inactivation. *Annual Review of Genetics.* **9**, 1-18.

Cattanach, B.M. & Papworth, D. (1981). Controlling elements in the mouse. V. Linkage tests with X linked genes. *Genetical Research.* **38**, 57-70.

Challoner, S. (1975). Studies of oogenesis and follicular development in the golden hamster. 3. The initiation of follicular growth *in vitro*. *J. Anat.* **119**, 157-162.

Chapman, V.M., Kratzer, P.G., Siracusa, L.D., Quarantillo, B.A., Evans, R., and Liskay, R.M. (1982). Evidence for DNA modification in the maintenance of X-chromosome inactivation of adult mouse tissues. *Proc. Natl. Acad. Sci. U.S.A.* **79**, 5357-5361.

Chatterjee, A., and Greenwald, G.S. (1972). The long-term effects of unilateral ovariectomy of the cycling hamster and rat. *Biol. Reprod.* **7**, 238-246.

Chiras, D.D. (1976). Studies on ovarian follicular kinetics and thymidine incorporation in the golden hamster (*Mesocricetus auratus*). Ph.D. Thesis. University of Kansas.

Chiras, D.D., and Greenwald, G.S. (1978). Acute effects of unilateral ovariectomy on follicular development in the cyclic hamster. *J. Reprod. Fert.* **52**, 221-225.

Clarke, I.J. and Cummins, J.T. (1984). Direct pituitary effects of estrogen and progesterone on gonadotropin secretion in the ovariectomized ewe. *Neuroendocrinology.* **39**, 267.

Collins, D.C. and Kent, H.A. Jr. (1964). Polynuclear ova and polyovular follicles in the ovaries of young guinea pigs. *Anat. Rec.* **148**, 115-119.

Cornfield, J., Steinfeld, J., and Greenhouse, S.W. (1960). Models for the interpretation of experiments using tracer compounds. *Biometrics* **16**, 212-234.

Corvol, P. and Bardin, C.W. (1973). Species distribution of testosterone-binding globulin. *Biol. Reprod.* **8**, 277-282.

Coulson, P.B. (1979). Characterization of polyploidy in ovarian granulosa cells. In. *Ovarian Follicular Development and Function.* . Midgley, A.R. & Sadler. W.A. Raven press, New York. pp. 385-394.

Cummins, J.M., and Woodall, P.F. (1985). On mammalian sperm dimensions. *J. Reprod. Fert.* **75**, 153-175.

Curry, Jr., T.E., Lawrence., Jr., I.E., and Burden, H.W. (1984). Effect of ovarian sympathectomy on follicular development during compensatory ovarian hypertrophy in the guinea pig. *J. Reprod. Fert.* **71**, 39-44.

Curtis, E.M. (1962). Normal ovarian histology in infancy and childhood. *Obstetrics and Gynecology.* **19**, 444-454.

David, G.F.X., Anand Kumar, T.C., and Baker, T.G. (1974). Uptake of tritiated thymidine by primordial germinal cells in the ovaries of the adult slender loris. *J. Reprod. Fert.* **41**, 447-451.

Davis, D.E. and Hall, O. (1950). Polyovuly and anovular follicles in the wild Norway rat. *Anat. Rec.* **107**, 187-192.

Dawson, A.B. (1951). Histogenetic interrelationships of oocytes and follicle cells. A possible explanation of the mode of origin of certain polyovular follicles in the immature rat. *Anat. Rec.* **110**, 181-197.

Dederer, P.H. (1934). Polyovular follicles in the cat. *Anat. Rec.* **60**, 391-396.

Dekel, N., Kraicer, P.F., Phillips, D.M., Sanchez, R.S., and Segal, S.J. (1978). Cellular associations in the rat oocyte cumulus-cell complex. Morphology and ovulatory changes. *Gamete Research.* **1**, 47.

De Jong, F.H., and Sharpe, R.M. (1976). Evidence for inhibin like activity in bovine follicular fluid. *Nature.* **263**, 71-72.

DePaolo, L.V., and Barraclough, C.A. (1979). Does dependent effects of Progesterone on the facilitation and inhibition of spontaneous gonadotropin surges in estrogen treated ovariectomised rats. *Biol. Reprod.* **21**, 1015-1023.

de Reviers, M.M. (1987). Hemi-ovariectomy and follicular growth in the immature rat. *Acta. Endocr.* **114**, 547-551.

Desaive, P. (1949). Étude des follicules a ovocytes doubles ou multiples dans l'ovaire de lapine adulte. *Arch. Biol.* **60**, 357-407.

diZerega, G.S. and Hodgen, G. (1979). Pregnancy associated ovarian refractoriness to gonadotropin: a myth. *Am. J. Obstet. Gynecol.* **134**, 819-822.

Driancourt, M.A., Cahill, L.P., and Bindon, B.M. (1985). Ovarian follicular populations and preovulatory enlargement in Booroola and control Merino ewes. *J. Reprod. Fert.* **73**, 93-107.

Drury, R.A.B. and Wallington, E.A. (1967). *Histological technique.* Oxford University Press. New York.

Dufour, J., Cahill, L.P., and Mauleon, P. (1979). Short- and long-term effects of hypophysectomy and unilateral ovariectomy on ovarian follicular populations in sheep. *J. Reprod. Fert.* **57**, 301-309.

Dullaart, J., Kent, J., and Ryle, M. (1975). Serum gonadotropin concentrations in infant female mice. *J. Reprod. Fert.* **43**, 189-192.

Edwards, R.G. (1970). Are oocytes formed and used sequentially in the mammalian ovary? *Phil. Trans. Roy. Soc. Lond B.* **259**, 103-105.

Edwards, R.G. (1974). Follicular fluid. *J. Reprod. Fert.* **37**, 189-219.

Edwards, R.G., Fowler, R.E., Gore-Langton, R.E., Gosden, R.G., Jones, E.C., Readhead, C., and Steptoe, P.C. (1977). Normal and abnormal follicular growth in mouse, rat and human ovaries. *J. Reprod. Fert.* **51**, 237-263.

Edwards, R.G. (1980). *Conception in the human female.* Academic Press. London.

Engle, E.T., (1927). Polyovular follicles and polynuclear ova in the mouse. *Anat. Rec.* **35**, 341.

Erickson, B.H. (1966). Development and senescence of the postnatal bovine ovary. *J. Anim. Sci.* **25**, 800-805.

Erickson, B.H. (1967). Radioresponse of the pre-puberal porcine ovary. *Int. J. Radiat. Biol.* **13**, 57-67.

Erickson, G.F., Hofeditz, C., Unger, M., Allen, W.R., and Dulbecco, R. (1985). A monoclonal antibody to a mammary cell line recognises distinct sub types of ovarian granulosa cells. *Endocrinology.* **117**, 1490-1499.

Eshkol, A. and Lunenfeld, B. (1972). Gonadotropic regulation of ovarian development in mice during infancy. In. *Gonadotropins.* Saxena, B.B., Belling, C.G., & Gandy, H.M. (Eds). Wiley. New York. pp 335-346.

Eshkol, A., Lunenfeld, B., and Peters, H. (1970). "Ovarian development in infant mice. Dependence on gonadotrophic hormones". In. *Gonadotrophins and Ovarian Development.* W.R. Butt. A.C. Crooke and M. Ryle (Eds). E. and S. Livingstone, Edinburgh. pp 249-258.

Evans, H.M. and Swezy, O. (1931). Ovogenesis and the normal follicular cycle in adult mammalia. *Mem. Univ. Calif.* **9**, 119.

Everett, J.W. (1948). Progesterone and estrogen in the experimental control of ovulation time and other features of the estrous cycle in the rat. *Endocrinology.* **43**, 389-405.

Faddy, M.J. (1976). A note on the general time dependent stochastic compartmental model. *Biometrics.* **32**, 443-448.

Faddy, M.J., and Jones, M.C. (1987). Fitting time-dependent multi-compartment models: a case study. *Biometrics* (Submitted for publication).

Faddy, M.J., Jones, E.C., and Edwards, R.G. (1976). An analytical model for ovarian follicle dynamics. *J. exp. Zool.* **197**, 173-185.

Faddy, M.J., Gosden, R.G. and Edwards, R.G. (1983). Ovarian follicle dynamics in mice: a comparative study of three inbred strains and an F_1 hybrid. *J. Endocr.* **96**, 23-33.

Fanjul, L.F., Ruiz de Galarreta, C.M., and Hsueh, A.J.W. (1983). Progestin augmentation of gonadotropin-stimulated progesterone production by cultured rat granulosa cells. *Endocrinology.* **112**, 405-407.

Fekete, E. (1950). Polyovular follicles in the C58 strain of mice. *Anat. Rec.* **108**, 699-707.

Finch, C.E., and Gosden, R.G. (1986). Animal models for the human menopause. In. *Aging, reproduction and the climacteric.* pp. 3-34. Mastroianni, L. Jr & Paulsen, C.A. (Eds). London: Plenum Press.

Findlay, J.K., and Cumming, I.A. (1977). The effect of unilateral ovariectomy on plasma gonadotropin levels, estrus and ovulation rate in sheep. *Biol. Reprod.* **17**, 178-183.

Fleming, M.W., Rhodes, R.C., and Dailey, R.A. (1984). Compensatory responses after unilateral ovariectomy in rabbits. *Biol. Reprod.* **30**, 82-86.

Fortune, J., and Vincent, S. (1983). Progesterone inhibits the induction of aromatase in rat granulosa cells in vitro. *Biol. Reprod.* **28**, 1078-1089.

Fraenkel, L. (1910). Die funktion des corpus luteum. *Arch. Gynaekol.* **68**, 438.

Fraser, H.M. (1983). LHRH agonist on stump tailed monkeys. *Endocrinology.* **112**, 245-253.

Fry, R.C., Clark, I.J. & Cahill, L.P. Cahill, (1987), Changes in gonadotrophin concentrations are not necessarily involved in ovarian compensation after unilateral ovariectomised in sheep. *J. Reprod. Fert.* **79**, 45-48.

Fukuda, M., Katayama, K., and Shimpei, T. (1980). Inhibitory effect of progesterone on follicular growth and induced super ovulation in the rat. *Arch. Gynecol.* **230**, 77-87.

Gans, C. (1979). Momentarily excessive construction as the basis for protoadaptation. *Evolution, Lawrence, Kans.* **33**, 227-233.

Garza, F., and Terranova, P.F. (1984). Inhibition of thecal androstenedione production by exogenous progesterone in the cyclic hamster. *J. Reprod. Fert.* **70**, 493-498.

Gérendai, I., Marchetti, B., Maugeri, S., Amico Roxas, M., and Scapagnini, U. (1978). Prevention of compensatory ovarian hypertrophy by local treatment with 6-OHDA. *Neuroendocrinology.* **27**, 272-278.

Gilula, N.B., Epstein, M.L., and Beers, W.H. (1978). Cell-cell communication and ovulation: A study of the cumulus-oocyte complex. *J. Cell. Biol.* **78**, 58-75.

Godfrey, K. (1983). *Compartmental Models and their Application.* London: Academic Press.

Goette, A. (1875). *"Die Entwickelungsgeschichte der Unke (Bombinator igneus) als Grundlage einer vergleichenden Morphologie der Wirbeltiere."* Leopold Voss, Leipzig.

Goff, A.K., Leung, P.C., and Armstrong, D.T. (1979). Stimulatory action of FSH and androgens on the responsiveness of rat granulosa cells to gonadotropins in vitro. *Endocrinology.* **104**, 1124-1129.

Goodman, A.L., and Hodgen, G.D. (1977). Systemic versus intraovarian progesterone replacement after luteectomy in rhesus monkeys; differential patterns of gonadotropins and follicular growth. *J. Clin. Endocrinol. Metab.* **45**, 837-840.

Goodman, R.L., Pickover, S.M., and Karsch, F.J. (1981). Ovarian feedback control of follicle-stimulating hormone in the ewe: evidence for selective suppression. *Endocrinology.* **108**, 772-.

Goldenberg, R.L., Vaitukaitis, J.L., and Ross, G.T. (1972). Estrogen and follicle stimulating hormone interactions on follicle growth in rats. *Endocrinology.* **90**, 1492-1498.

Gosden, R.G. (1985a). Maternal age: A major factor affecting the prospects and outcome of pregnancy. *Annals of the New York Academy of Sciences.* **442**, 45-54.

Gosden, R.G. (1985b). *Biology of menopause. The causes and consequences of ovarian ageing*. London: Academic Press.

Gosden, R.G., and Byatt-Smith, J.G. (1986). Oxygen concentration gradient across the ovarian follicular epithelium: model, predictions and implications. *Human Reprod.* **1**, 65-68.

Gosden, R.G., Laing, S.C., Flurkey, K., and Finch, C.E. (1983). Graafian follicle growth and replacement in anovulatory ovaries of ageing C57BL/6J mice. *J. Reprod. Fert.* **69**, 453-462.

Gougeon, A. (1981). Frequent occurrence of multiovular follicles and multinuclear oocytes in the adult human ovary. *Fertil. Steril.* **35**, 417-422.

Gould, S.J. (1966). Allometry and size in ontogeny and phylogeny. *Biol. Revs.* **41**, 587-640.

Govan, A.D.T. (1970). Ovarian follicular activity in late pregnancy. *J. Endocrinol.* **48**, 235-241.

Graham, C.E. and Bradley, C.F. (1971). Polyovular follicles in squirrel monkeys after prolonged diethyl-stilboestrol treatment. *J. Reprod. Fert.* **27**, 181-185.

Greenwald, G.S. (1961). Quantitative study of follicular development in the ovary of the intact or unilaterally ovariectomized hamster. *J. Reprod. Fert.* **2**, 351-361.

Greenwald, G.S. (1966). Ovarian follicular development and pituitary FSH and LH content in the pregnant rat. *Endocrinology.* **79**, 572-578.

Greenwald, G.S. (1973). Distribution between developing reserve follicles in the cycling hamster. *Ann. Biol. Bioch. Biophys.* **13**, 199-210.

Greenwald, G.S. (1974). Quantitative aspects of follicular development in the untreated and PMS-treated cyclic hamster. *Anat. Rec.* **178**, 139-144.

Greenwald, G.S. (1977). Exogenous progesterone: Influence on ovulation and hormone levels in the cyclic hamster. *J. Endocrinol.* **73**, 151-155.

Greenwald, G.S. (1978). Follicular activity in the mammalian ovary. In *The vertebrate ovary.* pp. 639-689. Jones, R.E. (Ed). Plenum press. New York.

Greenwald, G.S. and Choudary, J.B. (1969). Follicular development and induction of ovulation in the pregnant mouse. *Endocrinology.* **84**, 1512-1516.

Greenwald, G.S. Keever, J.E., and Grady, K.L. (1967). Ovarian morphology and pituitary FSH and LH concentration in the pregnant and lactating hamster. *Endocrinology.* **80**, 851-856.

Guthrie, M.J. and Jeffers, K.R. (1938). Ovaries of bat *Myotis lucifugus. lucifugus.* after injection of hypophyseal extract. *Anat. Rec.* **72**, 11-36.

Hage, A.J., Groen-Klevant, A.C., and Welschen, R. (1978). Follicle growth in the immature rat ovary. *Acta Endocrinol.* **88**, 375-382.

Halpin, D.M.G., Charlton, H.M., and Faddy, M.J. (1986). Effects of gonadotrophin deficiency on follicular development in hypogonadal (hpg) mice. *J. Reprod. Fert.* **78**, 119-125.

Harcourt, A.H., Harvey, P.H., Larson, S.G., and Short, R.V. (1981). Testis weight, body weight and breeding system in primates. *Nature, Lond.* **293**, 55-57.

Harlow, C.R., Hearn, J.P., and Hodges, J.K. (1984). Ovulation in the marmoset monkey: endocrinology, prediction and detection. *J.Endocr.* **103**, 17-24.

Harman, S.M., Louvet, J.P., and Ross, G.T. (1975). Interaction of estrogen and gonadotrophins on follicular atresia. *Endocrinology.* **96**, 1145-1152.

Harrison, R.J. (1948). The changes occurring in the ovary of the goat during the oestrous cycle and in early pregnancy. *J. Anat.* **82**, 21-48.

Harrison, R.J. (1949). Multiovular follicles in the ovaries of lower primates. *Nature, Lond.* **164**, 409-410.

Harrison, R.J. and Weir, B.J. (1977). Structure of the mammalian ovary. In. *The Ovary.* Lord Zuckerman & B.J. Weir (Eds). Academic Press, New York, San Francisco, London. Vol. I. pp. 113-217.

Harrison Matthews, L. (1937). The female sexual cycle in the British horseshoe bats, *Rhinolophus ferrum-equinum insulanus* Barrett-Hamilton and *R. hipposideros minutus* Montagu. *Trans. zool. Soc. Lond.* **23**, 224-255.

Hartman, C.G. (1926). Polynuclear ova and polyovular follicles in the opossum and other mammals with the special reference to the problem of fecundity. *The American Journal of Anatomy.* **37**, 1-52.

Hartshorne, G.M. (1987). Sub-populations of granulosa cells within the pre-ovulatory human follicle. *Human Reproduction.* Suppl. abstract No 168. Abstracts from 3rd meeting of ESHRE. IRL Press. Oxford.

Hay, M.F., and Moor, R.M. (1978). Changes in the Graafian follicle population during the follicular phase of the oestrous cycle. In. *Control of ovulation.* Crighton, D.B., Foxcroft, G.R., Haynes, N.B. & Lamming. (Eds). pp. 177-196. Butterworth. London.

Hemreck, A.S, and Greenwald, G.S. (1964). The effects of unilateral ovariectomy on follicular maturation in the guinea pig. *Anat. Rec.* **148**, 171-176.

Henderson, S.A. and Edwards, R.G. (1968). Chiasma frequency and maternal age in mammals. *Nature.* **218**, 22-28.

Hillier, S.G., and Ross, G.T. (1979). Effects of exogenous testosterone on ovarian weight, follicular morphology and intraovarian progesterone concentration in estrogen-primed hypophysectomized immature female rats. *Biol. Reprod.* **20**, 261-268.

Hirshfield, A.N., (1982). Follicular recruitment in long term hemicastrate rats. Biol. Reprod. 27, 48-53.

Hirshfield, A.N. (1983). Compensatory ovarian hypertrophy in the long term hemi castrate rats: size distribution of growing and atretic follicles. Biol. Reprod. 28, 271-278.

Hirshfield, A.N. (1986a). Effect of a low dose of PMSG on follicular recruitment and atresia in cycling rats. Biol. Reprod. 35, 113-118.

Hirshfield, A.N. (1986b). Patterns of [^{3}H] thymidine incorporation differ in immature rats and mature cycling rats. *Biol. Reprod.* **34**, 229-235.

Hirshfield, A.N., and Midgley, Jr., A.R. (1978), the role of FSH in the selection of large ovarian follicles in the rat. Biol. Reprod. 19, 606-611.

Hodges, J.K., Henderson, C., and M^{c}Neilly, A.S. (1983). Circulating oestrogen concentrations during pregnancy in the African elephant (*Loxodonta africana*). *J. Reprod. Fert.* **67**, 121-127.

Hoffman, J.C. and Schwartz, N.B. (1965). Timing of ovulation following progesterone withdrawal in the rat. *Endocrinology.* **76**, 626-631.

Hope, J. (1965). The fine structure of the developing follicle of the rhesus ovary. *J. Ultrastruct. Res.* **12**, 592-610.

Hunter, J. (1787). An experiment to determine the effect of extirpating one ovarium upon the number of young produced. *Phil. Trans. R. Soc. London.* **77**, 233-239.

Iguchi, T., Takasugi, N., Bern, H.A., and Mills, K.T. (1986). Frequent occurrence of polyovular follicles in ovaries of mice exposed neonatally to diethylstilbestrol. *Teratology.* **34**, 29-35.

Ingram, D.L. (1953). The effect of hypophysectomy on the number of oocytes in the adult albino rat. *J. Endocr.* **9**, 307-311.

Jeffcoate, S.L. (1986). Anterior pituitary hormones. In. *Scientific foundations of obstetrics and Gynaecology. Philipp, E.*, Barnes, J., & Newton, M. (Eds). Heinemann. Lond. pp. 443-448.

Johnson, S.K., Smith, M.F., and Elmore, R.G. (1985). Effect of unilateral ovariectomy and injection of bovine follicular fluid on gonadotropin secretion and compensatory ovarian hypertrophy in prepuberal heifers. *J. Anim. Sci.* **60**, 1055-1060.

Jones, E.C., and Krohn, P.L. (1960). The effect of unilateral ovariectomy on the reproductive lifespan of mice. J. Endocrinol. 20, 129-134.

Jones, E.C., and Krohn, P.L. (1961a). The relationships between age and numbers of oocytes and fertility in virgin and multiparous mice. *J. Endocrinol.* **21**, 469-495.

Jones, E.C., and Krohn, P.L. (1961b). The effect of hypophysectomy on age changes in ovaries of mice. *J. Endocrinol.* **21**, 497-509.

Jones, G.S. (1968). Induction of ovulation. *Ann. Rev. Med.* **19**, 351-372.

Kennedy, W.P. (1924). The occurrence of polyovular Graafian follicles. *J. Anat.* **58**, 328-334.

Kennedy, T.G., and Kennedy, J.P. (1972). Effects of age and parity on reproduction in young female mice. *J. Reprod. Fert.* **28**, 77-84.

Kent, H.A. Jr. (1958). Reduction of polyovular follicles and polynuclear ova by pituitary extracts. *Anat. Rec.* **132**, 463-464.

Kent, H.A. Jr. (1959). Reduction of polyovular follicles and polynuclear ova by oestradiol monobenzoate. *Anat. Rec.* **134**, 455-462.

Kent, H.A. Jr. (1960). Polyovular follicles and multinuclear ova in ovaries of young mice. *Anat. Rec.* **137**, 521-524.

Kent, H.A. Jr. (1962a). Polyovular follicles and multinuclear ova in ovaries of young rats. *Anat. Rec.* **142**, 25-29.

Kent, H.A. Jr. (1962b). Polyovular follicles and multinuclear ova in ovaries of young hamsters. *Anat. Rec.* **143**, 345-349.

Kent, H.A. Jr. (1964). Ovulation and fertilisation of abnormal ova of the golden hamster. *Fertil. Steril.* **15**, 591-596.

Kent, H.A. Jr. and Mandel, J.A. (1968). Correlation of ovarian abnormalities in young golden hamsters with blood estrogen content. *Anat. Rec.* **161**, 253-256.

Kent, H.A. Jr. and Mandel, J.A. (1970) Correlation of ovarian abnormalities in young golden hamsters with ovarian estrogen and progesterone. *Anat. Rec.* **168**, 233-236.

Kent, J. (1973). Some effects of steroids on the immature mouse ovary *in vitro*. *J. Reprod. Fertil.* **34**, 297-303.

Kim, I., and Greenwald, G.S. (1987). Stimulatory and inhibitory effects of progesterone on follicular development in the hypophysectomized follicle-stimulating hormone/luteinizing hormone-treated hamster. *Biol. Reprod.* **36**, 270-276.

Kölliker, A. von. (1898). Über die Markkanäle und Markstränge in den Eierstöcken junger Hündinnen. *Verhandlungen der anatomischen Gesellschaft.* **14**, 149-156.

Korenman, S.G., Stevens, R.H., Carpenter, L.A., Robb, M., Niswender, G.D., and Sherman, B.M. (1974). Estradiol radioimmunoassay without chromatography: procedure, validation and normal values. *J. Clin. Endocr. Metab.* **38**, 718-720.

Kozak, L.P., McLean, G.K., and Eicher, E.M. (1974). X linkage of phosphoglycerate kinase in the mouse. *Biochem. Genet.* **11**, 41-47.

Krarup, T., Pedersen, T., and Faber, M. (1969). Regulation of oocyte growth in the mouse ovary. *Nature.* **224**, 187-188.

Krohn, P.L. (1967). Factors influencing the number of oocytes in the ovary. *Arch. Anat. Microscop. Morphol. Exp.* **56**, 151-159.

Lacker, H.M. and Peskin, C.S. (1981). Control of ovulation number in a model of ovarian follicular maturation. *Lectures on Mathematics in the Life Sciences.* **14**, 21-57.

Land, R.B. (1973), Ovulation rate in Finn Dorset sheep following unilateral ovariectomy or chlorpromazine treatment at different stages of the oestrous cycle. *J. Reprod. Fert.* **33**, 99-105.

Lane, C.E. (1938). Aberrant ovarian follicles in the immature rat. *Anat. Rec.* **71**, 243-247.

Leach, B.J. and Conway, C.H. (1963). Origin and fate of polyovular follicles in the striped skunk. *J. Mammal.* **44**, 67-74.

Linkie, D.M., and Niswender, G.D. (1972). Serum levels of prolactin, luteinizing hormone, and follicle stimulating hormone during pregnancy in the rat. *Endocrinology.* **90**, 632-637.

Linkie, D.M., and La Barbera, A.R. (1979). Serum binding proteins in tissues of the immature rat: quantitation by RIA. *Proc. Soc. Exp. Biol. Med.* **161**, 7-12.

Lintern-Moore, S. (1972). Initiation of follicular growth in the infant mouse ovary by exogenous gonadotrophin. *Biol. Reprod.* **17**, 635-639.

Lintern-Moore, S. and Moore, G.P.M. (1978). The initiation of follicle and oocyte growth in the mouse ovary. *Biol. Reprod.* **20**, 773-778.

Lipschutz, A. (1927). On some fundamental laws of ovarian dynamics. *Biol. Rev.* **2**, 263-280.

Lipschutz, A. (1928). New developments in ovarian dynamics and the law of follicular constancy. *Brit. J. Exp. Biol.* **5**, 283-291.

Lloyd, R.S., and Rubenstein, B.B. (1941). Multiple ova in the follicles of juvenile monkeys. *Endocrinology.* **29**, 1008-1014.

Loeb, L. (1917). The concrescence of follicles in the hypotypical ovary. *Biol. Bull., Wood's Hole.* 32, 187.

Luthardt, F.W., Palmer, C.G. and Yo, P.L. (1973). Chiasma and univalent frequencies in aging female mice. *Cytogenetics and Cell Genetics.* 12, 68-79.

Lyon, M.F. (1974) Mechanisms and evolutionary origins of variable X-chromosome activity in mammals. *Proc. R. Soc.* Series B: Biological Sciences, 187, 243-268.

McCormack, J.T., and Greenwald, G.S. (1974). Progesterone and oestradiol-17β concentrations and its relationship to serum follicle-stimulating hormone in immature female rats. *J. Endocrinol.* 62, 101-107.

McLaren, A. (1966), Regulation of ovulation rate after removal of one ovary in mice. *Proc. R. Soc.* Series B: Biological Sciences. 166, 316-340.

M^{c}Natty, K.P. (1978). Follicular fluid. In. *The Vertebrate Ovary.* Jones, R.E. (Ed). Plenum press. New York. Ch.7. pp. 215-260.

Mainland, D. (1927). The pluriovular follicle, with reference to its occurrence in the ferret. *J. Anat.* 62, 139-158.

Mallampati, R.S., and Casida, L.E. (1970). Ovarian compensatory hypertrophy following unilateral ovariectomy during the breeding season in the ewe. *Biol. Reprod.* 3, 43-46.

Mandl, A.M. and Shelton, M. (1959). A quantitative study of oocytes in young and nulliparous laboratory rats. *J. Endocrinol.* 18, 444-450.

Mandl & Zuckerman, S. (1950), The numbers of normal and atretic oocytes in the mature rat. *J. Endocrinol.* 6, 426-435.

Mandl, A.M. and Zuckerman, S. (1951). Numbers of normal and atretic oocytes in unilaterally spayed rats. *J. Endocrinol.* 7, 112-119.

Mandl & Zuckerman, S. (1952), Cyclical changes in the number of medium and large follicles in the adult rat ovary. *J. Endocrinol.* **8**, 341-346.

Marois, M. and Buvet, M. (1972). Experimental increase with anti-estrogen in number of polyovular follicles in rabbit ovary. *Cr. Ac. Sci. D.* **274**, 2704.

Matis, J.H., and Hartley, H.O. (1971). Stochastic compartmental analysis: model and least squares estimation from time series data. *Biometrics.* **27**, 77-102.

May, R.M. and Rubenstein, D.I. (1984). Reproductive strategies. In. *Reproduction in mammals. Book 4. Reproductive fitness:* pp. 1-23. 2nd edn. Austin, C.R. & Short, R.V. (Eds). Cambridge: Cambridge University Press.

Merchant-Larios, H. (1976). The role of germ cells in the morphogenesis and cytodifferentiation of the rat ovary. in: *Progress in Differentiation Research* (N. Müller-Bérat, ed.), pp. 453-462. North Holland, Amsterdam.

Merchant-Larios, H. (1979). Origin of the somatic cells in the rat gonad: an autoradiographic approach. *Ann. Biol. Anim. Biochim. Biophys.* **19**, 1219-1299.

Micklem, H.S., Lennon, J.E., Ansell, J.D., and Gray, R.A. (1987). Numbers and dispersion of repopulating hematopoietic cell clones in radiation chimeras as functions of injected cell dose. *Exp. Hematol.* **15**, 251-257.

Monk, M. and Harper, M.I. (1979). sequential X-chromosome inactivation coupled with cellular differentiation in early mouse embryos. *Nature.* **281**, 311-313.

Moor, R.M. (1983). Contact, signalling and cooperation between follicle cells and dictyate oocytes in mammals. In. *Current problems in Germ Cell Differentiation*: pp 307-326. McLaren, A. & Wylie, C.C. (Eds). Cambridge: Cambridge University Press.

Moore, P.J., and Greenwald, G.S. (1974). Effect of hypophysectomy and gonadotropin treatment on follicular development and ovulation in the hamster. *Am. J. Anat.* **139**, 37-48.

Moss, G.E., Crowder, M.E., and Nett, T.M. (1981). GnRH-Receptor interaction, VI. Effect of progesterone and estradiol on hypophyseal concentrations of gonadotrophins. *Biol. Reprod.* **25**, 938-

Mossman, H.W., and Duke, K.L. (1973) *Comparative Morphology of the Mammalian Ovary*. University of Wisconsin Press.

Motta, P. (1974) Superficial epithelium and surface invaginations in the cortex of mature rabbit ovaries: a note on the histogenesis of the interstitial cells. *Fertil. Steril.* **25**, 336-47

Motta, P. and Makabe, S. (1982). Development of the ovarian surface and associated germ cells in the human fetus. *Cell Tissue Res.* **226**, 493-510.

Murr, S.M., Bradford, G.E., and Geschwind, I.I. (1974a). Plasma luteinizing hormone, follicle stimulating hormone and prolactin during pregnancy in the mouse. *Endocrinology*. **94**, 112-116.

Murr, S.M., Stabenfeldt, G.H., Bradford, G.E., and Geschwind, I.I. (1974b). Plasma progesterone during pregnancy in the mouse. *Endocrinology*. **94**, 1209-1211.

Nadler, R.D., Graham, C.E., Collins, D.C., and Gould, K.G. (1979). Plasma gonadotropins, prolactin, gonadal steroids, and genital swelling during the menstrual cycle of lowland gorillas. *Endocrinology*. **105**, 290-296.

Nandedkar, T.D. (1981). Is Dihydrotestosterone a cause of follicular atresia? *Medical hypotheses*. **7**, 801-808.

Nelson, J.F., Felicio, L.S., Randall, P.K., Sims, C. and Finch, C.E. (1982). A longitudinal study of estrous cyclicity in aging C57BL/6J mice: I. Cycle frequency, length and vaginal cytology. *Biol. Reprod.* **27**, 327-339.

Nelson, Gosden, R.G. and Felicio, (1985). Effect of dietary restriction on estrous cyclicity and follicular reserves in aging C57 BL/6J mice. *Biol. Reprod.* **32**, 515-522.

Nicoll, M.E. and Racey, P.A. (1985). Follicular development, ovulation, fertilisation and fetal development in tenrecs (*Tenrec ecaudatus*). *J. Reprod. Fert.* **74**, 47-55.

Nielsen, J.T. and Chapman, V.M. (1977). Electrophoretic variation for X-chromosome-linked phosphoglycerate kinase (PGK-1) in the mouse. *Genetics.* **87**, 319-325.

Nieuwkoop, P.D. (1949). The present state of the problem of the «Keimbahn» in vertebrates. *Experientia.* **5**, 308-312.

Noden, P.A., Oxender, W.D., and Hafs, H.D. (1975). The cycle of oestrus, ovulation and plasma levels of hormones in the mare. *J. Reprod. Fert. (Suppl.)* **23**, 189-192.

Norris, M.L. and Adams, C.E. (1982). Effect of unilateral ovariectomy on the population of ovarian follicles relative to age in the Mongolian gerbil (*Meriones unguiculatus*). *J. Reprod. Fert.* **66**, 335-340.

Numazawa, A., and Kawashima, S. (1982). Morphometric studies on ovarian follicles and corpora lutea during the oestrous cycle in the mouse. *J. Reprod. Fert.* **64**, 275-283.

Nussbaum, M. (1880). Zur Differenzierung des Geschlechts im Theirreich. *Arch. Mikrosk. Anat.* **18**, 1-120.

O'Donoghue, (1912). The corpus luteum in the non-pregnant Dasyurus and polyovular follicles in Dasyurus. *Anat. Anz.* **41**,

O, W.S. and Baker, T.G. (1976). Initiation and control of meiosis in hamster gonads in vitro. *J. Reprod. Fertil.* **8**, 339-401.

Oakberg, E.F. (1979). Follicular growth and atresia in the mouse. *In Vitro.* **15**, 41-49.

Ohno, S. and Smith, J.B. (1964). Role of fetal follicular cells in meiosis of mammalian oocytes. *Cytogenetics.* **3**, 324-333.

Okkelberg, P. (1921). The early history of the germ cells in the brook lamprey *Entosphenus wilderi* (Gage), up to and including the period of sexual differentiation. *J. Morphol.* **35**, 1-151.

Osman, P. (1985). Rate and course of atresia during follicular development in the adult cyclic rat. *J. Reprod. Fert.* **73**, 261-270.

Osman, P. (1986). Morphometric analysis of follicular dynamics in pregnant and pseudopregnant rats. *J. Reprod. Fert.* **76**, 11-22.

Ota, T. (1934). Polyovular follicles in dogs. *Jap. Journal. Obst. Gynecol.* **17**, 207-209.

Otani, T., and Sasamoto, S. (1982). Plasma and pituitary hormone changes and follicular development after unilateral ovariectomy in cyclic rats. *J. Reprod. Fert.* **65**, 347-353.

Pankratz, D.S. (1938). Some observations of the Graafian follicles in an adult human ovary. *Anat. Rec.* **71**, 211.

Papadaki, L. (1978). Binovular follicles in the adult human ovary. *Fertil. Steril.* **29**, 342-350.

Parkes, A.S. (1932). Size of the Graafian follicle at ovulation. *Proc. R. Soc.* (B) **109**, 185-213.

Pearl, R., and Schoppe, W.F. (1921). Studies on the physiology of reproduction in the domestic fowl. **XVIII.** Further observations on the anatomical basis of fecundity. *J. Exp. Zool.* **34**, 101-118.

Pearson, O.P. (1949). Reproduction in a South American rodent, the mountain viscacha. *Am. J. Anat.* **84**, 143-173.

Pedersen, T. (1969) Follicle growth in the immature mouse ovary. *Acta Endocrinol.* **62**, 117-132.

Pedersen, T. (1970a). Follicle kinetics in the ovary of the cyclic mouse. *Acta Endocrinol.* **64**, 304-323.

Pedersen, T. (1970b). Determination of follicle growth rate in the ovary of the immature mouse. *J. Reprod. Fert.* **21**, 81-93.

Pedersen, T. (1972). Follicle growth in the mouse ovary. In *Oogenesis.* Biggers, J.D. & Schuetz, A.W. (Eds). University Park Press, Baltimore. Butterworth, London. pp. 261-276.

Pedersen, T. and Hartmann, N.R. (1971). The kinetics of granulosa cells in developing follicles in the mouse ovary. *Cell. Tissue. Kinet.* **4**, 171-184.

Pedersen, T., and Peters, H. (1968). Proposal for a classification of oocytes and follicles in the mouse ovary. *J. Reprod. Fert.* **17**, 555-557.

Pedersen, T., and Peters, H. (1971). Follicle growth and cell dynamics in the mouse ovary during pregnancy. *Fertil. Steril.* **22**, 42-52.

Peluso, J.J. and Steger, R.W. (1978). Role of FSH in regulating granulosa cell divisions and follicular atresia in rats. *J. Reprod. Fertil.* **54**, 275-278.

Peppler, R.D.& Greenwald,G.S.(1970a), Effects of unilateral ovariectomy on ovulation and cycle length in 4 and 5 day cycling rats. *Am. J.Anat.* **127**, 1-8.

Peppler, R.D.& Greenwald,G.S.(1970b), Influence of unilateral ovariectomy on follicular development in cycling rats. *Am. J.Anat.* **127**, 9-14.

Peppler, R.D., (1971), Effects of uni ovx on follicular development and ovulation in cycling ageing rats. *Am. J. Anat.* **132**, 423-427.

Peters, H. (1976). The development and maturation of the ovary. *Annls Biol. anim. Biochim. Biophys.* **16**, 271-278.

Peters, H. (1978). "Folliculogenesis in mammals" in *The Vertebrate Ovary.* R.E. Jones (Ed). Plenum Press, New York. pp. 121-144.

Peters, H. (1979). Some aspects of early follicular development. In *Ovarian follicular development and function.* pp 1-13. Midgley, A.R. and Sadler, W.A. (Eds). Raven Press. New York.

Peters, H., and Crone, M. (1967). DNA synthesis in oocytes of mammals. *Arch. Anat. Microsc. Morphol. Exp.* **56**, 160-170.

Peters, H., and Levy, E. (1966). Cell dynamics of the ovarian cycle. *J. Reprod. Fert.* **11**, 227-236.

Peters, H., Byskov, A.G., and Faber, M. (1973a). "Intraovarian regulation of follicle growth in the immature mouse ". In: *The Development and Maturation of the ovary and its Function,* H.Peters (Ed). Amsterdam: Excerpta Med., pp. 20-23.

Peters, H., Byskov, A.G., Lintern-Moore, S., Faber, M., and Andersen, M. (1973b). The effect of gonadotrophin on follicle growth initiation in the neonatal mouse ovary. *J. Reprod. Fertil.* **35**, 139-141.

Peters, H., Byskov, A.G., Himelstein-Braw, R., and Faber, M. (1975). Follicle growth: the basic event in the mouse and human ovary. *J. Reprod. Fert.* **45**, 559-566.

Philibert, D., Ojasoo, T., and Raynaud, J.-P. (1977). Properties of the cytoplasmic progestin-binding protein in the rabbit uterus. *Endocrinology.* **101**, 1850-

Pincus, G., and Chang, M.C. (1953). Effects of progesterone and related compounds on ovulation and early development in rabbit. *Acta Physiol. Lat. Am.* **3**, 177-183.

Polani, P.E. and Jagiello, G.M. (1976). Chiasmata, meiotic univalents and age in relation to aneuploid imbalance in mice. *Cytogenetics and Cell Genetics.* **16**, 505-529.

Ramirez, V.D., and Sawyer, C.H. (1974). Sex difference in the rat pituitary FSH response to unilateral gonadectomy as revealed in plasma radioimmunoassay. *Endocrinology.* **94**, 475-482.

Read, K.L.Q., Berry, P.J.B., Mariana, J.C. and de Reviers, M.M. (1981). Statistical modelling of the genesis and growth of ovarian follicles in infant rats. *Development and Function of Reproductive Organs.* Byskov, A.G. & Peters, H. (Eds). pp.279-288.

Redmer, D.A., Christenson, R.K., Ford, J.J. and Day, B.N. (1984). Effect of follicular fluid treatment on FSH and compensatory hypertrophy in prepubertal gilts. *Biol. Reprod.* **32**, 111-119.

Reyes, F.I., Winter, J.S.D., Faiman, C., and Hobson, W.C. (1975). Serial serum levels of gonadotropins, prolactin and sex steroids in the nonpregnant and pregnant chimpanzee. *Endocrinology.* **96**, 1447-1455.

Richards, J.S. (1979). Hormonal Control of Ovarian Follicular Development: A 1978 Perspective. *Rec. Prog. Horm. Res.* **35**, 343-373.

Richards, J.S. (1980). Maturation of Ovarian Follicles: Actions and Interactions of Pituitary and Ovarian Hormones on Follicular Cell Differentiation. *Physiological Reviews.* **60**, 51-89.

Richards, J.S. and Kersey, K.A. (1979). Changes in theca and granulosa cell function in antral follicles developing during pregnancy in the rat: gonadotrophin receptors, cyclic AMP and estradiol 17β. *Biol. Reprod.* **21**, 1185-1201.

Richards, J.S., and Bogovich, K. (1982). Effects of human chorionic gonadotropin and progesterone on follicular development in the immature rat. *Endocrinology.* **111,** 1429-1438.

Richards, J.S., Jonassen, J.A., and Kersey, K.A. (1980). Evidence that changes in tonic luteinizing hormone secretion determine the growth of preovulatory follicles in the rat. *Endocrinology.* **107,** 641-

Rudkin, G.T., and Griech, H.A. (1962). Persistence of oocyte nuclei from fetus to maturity in the laboratory mouse. *J. Cell Biol.* **12,** 169-75.

Russell, J.A. (1983). Precise measurement by image-shearing of nucleolar size in immunocytochemically identified peptidergic neurones. *J. Physiol.***346,** 3P.

Ryle, M. (1972). The growth *in vitro* of mouse ovarian follicles of different sizes in response to purified gonadotrophins. *J. Reprod. Fert.* **30,** 395-405.

Sacher, G.A. (1959). Relation of lifespan to brain weight and body weight in mammals. Ciba Fdn Coll. **5,** 115-141.

Sahu, A. (1985). Mitotic activity and its 24 hour rhythm in the ovarian follicles during the estrous cycle of a wild rat, *Bandicota bengalensis. Acta anat.* **123,** 195-200.

Saiduddin, S., Rowes, R.F. & Casida, L.E. (1970). Ovarian follicular changes following unilateral ovariectomy in the cow. *Biol. Reprod.* **2,** 408-412.

Sandberg, I.W. (1978). On the mathematical foundations of compartmental analysis in biology, medicine and ecology. *IEEE Trans. Circuits Syst.* CAS-**25,** 273-279.

Scaramuzzi, R.J., Caldwell, B.V., and Moor, R.M. (1970). Radioimmunoassay of LH and oestrogen during the oestrous cycle of the ewe. *Biol. Reprod.* **3,** 110-119.

Schmidt-Nielsen, K. (1984). *Scaling: Why is animal size so important?* Cambridge University Press. Cambridge.

Schreiber, J.R., and Hsueh, A.J.W. (1979). Progesterone "receptor" in rat ovary. *Endocrinology.* **105**, 915-919.

Schreiber, J.R., Nakamura, K., and Erickson, G.F. (1980). Progestins inhibit FSH steroidogenesis in cultured rat granulosa cells. *Mol. Cell. Endocr.* **19**, 165-173.

Schwall, R.H. and Niswender, G.D. (1985). Two types of steroidogenic luteal cells in the ewe: morphological and biochemical characteristics. In *Implantation of the human embryo*: pp. 31-38. Edwards, R.G., Purdy, J.M., and Steptoe, P.C. (Eds). London & Orlando: Academic Press.

Schwartz, N.B., and Tally, W.L. (1968). Daily measurements of pituitary LH content during pregnancy in the rat: do cyclic changes persist? *J. Reprod. Fertil.* **15**, 39-45.

Schwartz, N.B., Andersen, C.H., Neguin, L.G., and Ely, C.A. (1974). Follicular maturation. In *The control of the onset of puberty.* Grumbach, M.M., Grave, G.D. & Mayer, F.E. (Eds). pp. 367-385.

Setty, S.L., and Mills, T.M. (1987). The effects of progesterone on follicular growth in the rabbit ovary. *Biol. Reprod.* **36**, 1247-1252.

Shehata, R. (1974). Polyovular Graafian follicles in a new born kitten with a study of polyovuly in the cat. *Acta. Anat.* **89**, 21-30.

Sheppard, C.W. (1948). The theory of transfers within a multicompartment system using isotopic tracers. *Journal of Applied Physics.* **19**, 70-76.

Sheppard, C.W. and Householder, A.S. (1951). The mathematical basis of the interpretation of tracer experiments in closed steady state systems. *Journal of Applied Physics.* **22**, 510-520.

Siegel, S. (1956). *Non-parametric statistics for the behavioral sciences.* M^{c}Graw-Hill. International.

Siiteri, P.K., Murai, J.T., Hammond, G.L., Nisker, J.A., Raymoure, W.J. and Kuhn,R.W. (1982). The serum transport of steroid hormones. *Rec. Prog. Horm. Res.* **38**, 457-503.

Silverman, B.W. (1985). Some aspects of the spline smoothing approach to non-parametric curve fitting. *J. Roy. Stat. Soc. B.* **47**, 1-52.

Simkins, C.S. (1923). On the origin and migration of the so-called primordial germ cells in the mouse and rat. *Acta. Zool. Stockh.* **4**, 241-278.

Smith, M.S., Freeman, M.E., and Neill, J.D. (1975). The control of progesterone secretion during the estrous cycle and early pseudopregnancy in the rat: prolactin, gonadotropin and steroid levels associated with rescue of the corpus luteum of pseudopregnancy. *Endocrinology.* **96**, 219-226.

Snell, O. (1891). Das Gewicht des Gehirnes und des Hirnmantels der Säugethiere in Fähigkeiten. *Sitzungberichte der Gesellschaft für Morphologie und Physiologie in München.* **7**, 90-94.

Sokal, R.R. and Rohlf, F.J. (1981). Biometry: *The principles and practice of statistics in biological research.* W.H. Freeman and Company. San Francisco.

Speed, R.M. (1977). Effects of aging on meiotic chromosomes of male and female mice. *Chromosoma.* **63**, 241-254.

Stahl, W.R. (1967). Scaling of respiratory variables in mammals. *J. Appl. Physiol.* **22**, **453-460.**

Stebbings, R.E. (1977). Order Chiroptera, Bats. In *The handbook of British mammals*: pp. 68-128. Corbet, G.B. & Southern, H.N. (Eds). Oxford: Blackwells.

Stein, L.E. and Anderson, E. (1979). A qualitative and quantitative study of rete ovarii development in the fetal rat: correlation with the onset of meiosis and follicle cell appearance. *Anat. Rec* **193**, 197-211.

Stiff, M.E., Bronson, F.H., and Stetson, M.H. (1974). Plasma gonadotropins in prenatal and prepubertal female mice: disorganization in the absence of a male. *Endocrinology.* **94,** 492-496.

Stockard, A.H. (1937). Studies on the female reproductive system of the prairie dog, Cynomys leucurus. 2. Normal cyclic phenomena of the ovarian follicles. *Pap. Mich. Acad. Sci.* **22,** 671.

Stone, M. (1984). Variance-Covariance modelling with chromosome markers. *J. Theor. Biol.* **107,** 275-286.

Swyte, D.A. and Rosberry, F.W. (1977). A comparison of some optical microscope mesurements of photomask linewidths. *Solid State Technol.* **August,** 70-75.

Szöllösi, D. (1978). Desmosomes: Their occurrence between adjacent primary oocytes in polyovular follicles in the rabbit. *Cell. Tissue. Res.* **191,** 115-119.

Talbert, G.B. (1978). Effect of aging of the ovaries and female gametes on reproductive capacity. In *Aging, Vol.4. The aging reproductive system.* pp. 59-83. Schneider, E.L. (Ed). Raven Press. New York.

Taya, K., Terranova, P.F., and Greenwald, G.S. (1981). Acute effects of exogenous progesterone on follicular steroidogenesis in the cyclic rat. *Endocrinology.* **108,** 2324-2330.

Tonetta, S.A., and diZerega, G.S. (1986). Paracrine regulation of follicular maturation in primates. *Clinics in Endocrinology and Metabolism.* **15,** 135-157.

Tripp, H.R.H. (1971). Reproduction in elephant-shrews (Macroscelididae) with reference to ovulation and implantation. *J. Reprod. Fertil.* **26,** 149-159.

Ullmann, S.L. and Dairi, M. (1986). Ovarian development and polyovular follicles in the potoroo. (Macropodidae, Marspialia). Abstract No. 58. Annual Conference of the Society for the Study of Fertility.

Vanhems, E., and Bousquet, J., (1973). Étude expérimentale chez le rat d'ovaires rendus dysgénésiques durant leur développement embryonnaire. *Ann. Biol. Anim. Biochem. Biophys.* **13**, 79-88.

Virgo, B.B., and Bellsard, G.D. (1974). Serum progesterone levels in the pregnant and post partum laboratory mouse. *Endocrinology.* **95**, 1486-1490.

Von Baer, K.E. (1827). "De ovi mammalium et hominis genesi". *Epistola ad Acad. Imper. Sci. petropolitanam.* L. Vossi Leipzig.

Waldeyer, W. (1870) *Eierstock und Ei.* Wilh. Engelmann, Leipzig.

Wareham, K.E., Lyon, M.F., Glenister, P.H., and Williams, E.D. (1987). Age related reactivation of an X-linked gene. *Nature.* **327**, 725-727.

Weick, R.F., Dierschke, D.J., Karsch, F.J., Butler, W.R., Hotchkiss, J., and Knobil, E. (1973). Periovulatory time courses of circulating gonadotropic and ovarian hormones in the rhesus monkey. *Endocrinology.* **93**, 1140-1147.

Weir, B.J. (1971). The reproductive organs of the female plains viscacha, *Lagostomus maximus*. *J. Reprod. Fert.* **25**, 365-373.

Weiss, L. (1971). Additional evidence of gradual loss of germ cells in the pathogenesis of streak ovaries in Turner's syndrome. *J. Med. Genet.* **8**, 540-544.

Welschen, R., and Dullaart, J. (1974). Serum concentrations of follicle stimulating hormone and luteinizing hormone after unilateral ovariectomy in the adult rat. *J. Endocrinol.* **63**, 421-422.

Welschen, R., Hermans, W.P., Dullart, J., and de Jong, F.H. (1977). Effects of an inhibin like factor present in bovine and porcine follicular fluid on gonadotrophin levels in ovariectomised rats. *J. Reprod. Fert.* **50**, 129-131.

Zoller, L.C. and Enelow, R. (1983). A quantitative histochemical study of lactate dehydrogenase and succinate dehydrogenase activities in the membrana granulosa of the ovulatory follicle of the rat. *Histochem. J.* **15**, 1055-1064.

Zoller, L.C. and Weisz, J. (1978). Identification of cytochrome p-450, and its distribution in the membrana granulosa of the preovulatory follicle, using quantitative cytochemistry. *Endocrinology* **103**, 310-313.

Zuckerman, S. (1951). The number of oocytes in the mature ovary. *Recent Prog. Horm. Res.* **6**, 63-109.

Zybina, E.V. and Grishche, T.A. (1980). Comparative study of normal and degenerating oocytes of the golden hamster at early post-natal development: Polyovular follicles and multinuclear oocytes. *Tsitologiya.* **22**, 121.

Appendix I:

Published papers and work related to thesis presented at meetings.

Work presented at meetings:

Society for the Study of Fertility (SSF) summer meeting 1984, Reading.

Evelyn Telfer, J.D. Ansell & R.G. Gosden.

A method for estimating the number of clones in the granulosa cell population of murine ovarian follicles.

SSF summer meeting 1985, Aberdeen.

Evelyn Telfer, R.G. Gosden & M.J. Faddy:

Follicular dynamics in mammalian ovaries.

R.G. Gosden & Evelyn Telfer.

Size and numbers of ovarian follicles and oocytes and their allometric relationships in mammals.

SSF summer meeting 1986, Bristol.

Evelyn Telfer & R.G. Gosden.

Polyovular follicles in the domestic bitch.

Publications:

Evelyn Telfer & R.G. Gosden, 1987.

A quantitative cytological study of polyovular follicles in mammalian ovaries with particular reference to the domestic bitch (*Canis familiaris*). *J. Reprod. Fert.* **81**, 137-147.

R.G. Gosden & Evelyn Telfer, (1987).
Numbers of follicles and oocytes in mammalian ovaries and their allometric relationships. *Journal of Zoology* 211, 169-175.

R.G. Gosden & Evelyn Telfer, (1987).
Scaling of follicular sizes in mammalian ovaries. *Journal of Zoology* 211, 157-168.

J. Zool., Lond. (1987) **211**, 169–175

Numbers of follicles and oocytes in mammalian ovaries and their allometric relationships

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(With 2 figures in the text)

The numbers of ovarian follicles present in the reserve pool of non-growing ('primordial') stages at the commencement of adult life in 19 species varied allometrically with both body weight (M, in kg) and with maximum life expectation (L, in years), the mathematical expressions being $27700M^{0.47}$ and $820M^{1.58}$, respectively. These allometric relationships, which could not be accounted for by differences in reproductive behaviour patterns or ovulation rates, indicate that species differences in the size of the follicular store, perhaps accompanied by more parsimonious utilization, could be a life strategy which guarantees fecundity throughout most of the lifespan. The number of follicles in humans at menarcheal age is commensurate with body size, and follicular deficiency at menopause in mid-life may have therefore arisen adventitiously with lifespan extension.

Contents

Introduction

The processes of oogenesis and follicle formation in mammalian ovaries have been completed by birth or shortly thereafter. The dynamics of these developmental processes and of subsequent follicle utilization are therefore expected to ensure that individuals can eke out their limited store of germ cells throughout life, whilst providing sufficient flexibility for fertile cycles to continue under favourable external conditions.

There is a large body of knowledge showing the effects of scaling on physiological parameters, many of which vary with body weight (M) according to the allometric formula $y = aM^b$; but there is little information about scaling of factors relating to reproductive system ageing (Calder, 1984). The present study was designed to test: (a) whether the provision of follicles for adult life varies allometrically with body weight and (b) the relationships between the numbers of follicles and body weight on the one hand and the maximum life expectation of the species on the other. To achieve a uniform basis for comparisons, the follicular populations of peripubertal ovaries have been studied, since developmental states at puberty are comparable in different species and only follicles which are potentially capable of contributing to fecundity will be present.

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TABLE I

List of species and their ovarian parameters

Code no.	Species	Order	No. primordial follicles*	No. growing follicles*	Growing fraction**	Modal ovulation rate
1	Bandicoot (*Isoodon macrourus*)	Marsupialia	12440	915	0·049	4
2	Common shrew (*Sorex araneus*)	Insectivora	3560	—	—	8
3	Pipistrelle bat (*Pipistrellus pipistrellus*)	Chiroptera	3268	254	0·072	1
4	Greater horseshoe bat (*Rhinolophus ferrumequinum*)	,,	7950	—	—	1
5	House mouse (*Mus musculus*)	Rodentia	4270	676	0·165	8
6	Wood mouse (*Apodemus sylvaticus*)	,,	3170	164	0·032	6
7	Bank vole (*Clethrionomys glareolus*)	,,	4380	269	0·056	4
8	Field vole (*Microtus agrestis*)	,,	2858	179	0·050	4
9	Norway rat (*Rattus norvegicus*)	,,	5180	—	—	10
10	Guinea pig (*Cavia porcellus*)	,,	29200	—	—	4
11	European rabbit (*Oryctolagus cuniculus*)	Lagomorpha	75120	3222	0·040	8
12	Domestic cat (*Felis catus*)	Carnivora	74520	1132	0·021	4
13	Domestic dog (*Canis familiaris*)	,,	150380	29775	0·198	4
14	Sheep (*Ovis aries*)	Artiodactyla	105450	475	0·007	1
15	Swine (*Sus scrofa*)	,,	420000	—	—	10
16	Cattle (*Bos taurus*)	,,	210000	—	—	1
17	Common marmoset (*Callithrix jacchus*)	Primates	17220	1877	0·109	2
18	Rhesus monkey (*Macaca mulatta*)	,,	100000	16100	0·161	1
19	Human (*Homo sapiens*)	,,	302000	12090	0·040	1

* Mean number per pair of ovaries

** Ratio of the number of growing follicles to the size of the total follicular population

Materials and methods

Data were obtained from non-pregnant representatives of 19 species among 8 orders (see Table I). Ovaries were obtained either at autopsy or at ovariectomy and prepared as serial histological sections 8 μm thick and stained with H. & E. They were collected without regard to the stages of the sexual cycle since the numbers of primordial follicles vary independently and there is the practical difficulty of finding a comparable stage among the variety of patterns, which included reflex ovulation in some species.

The average number of small, non-growing ('primordial') follicles in each species was obtained by counting in every 10th, 20th or 40th section (depending on organ size) for 4–8 individuals. Either the nucleus or, when single and distinctive, the nucleolus, was used as a marker and the total numbers of follicles were estimated by multiplying by both the sampling frequency and a correction factor (Abercrombie, 1946). Growing follicles were counted in the same sections, these being defined by those ranging from unilaminar follicles with a growing oocyte and cuboidal epithelium to Graafian sizes. Atretic follicles were included in the counts when the marker remained distinct. In the rare cases of binovular and polyovular follicles, it was the follicle rather than the enclosed oocytes that was counted. An attempt was made, however, to reduce this additional source of variation by choosing individuals and breeds in which the body size was close to that characteristic of the species. Average values for body weight (M, in kg) and ovulation rate at young adult ages were obtained from either unpublished or published sources. In most species, there was sufficient reliable data for estimating the maximum adult lifespan by subtracting the characteristic pubertal age from total longevity.

Data for the following 5 species were extracted from studies in which comparable cytological methods have been used. Those for domestic swine and cattle were obtained from Erickson (1966, 1967). In laboratory mice (strain A) and rats and humans, the numbers of follicles at pubertal ages were obtained from regression lines, which were based on large sets of data which extended across most of the lifespan (Block, 1952; Mandl & Shelton, 1959; Jones & Krohn, 1961). The regressions were obtained using an exponential model, $N = Ae^{-bt}$ since this produced a good fit with each data set (coefficients of determination, $r^2 = 0{\cdot}94$, $0{\cdot}80$, $0{\cdot}49$, respectively) and facilitated estimation of follicle numbers at puberty and the fractional disappearance rates for small follicles in adult life.

Results and discussion

The species characteristic number of follicles per individual at the onset of fertile life varied hypoallometrically, the body weight exponent being $0{\cdot}475 \pm 0{\cdot}038$ (Fig. 1). Since r^2 was 0·91, most interspecific variation was accounted for by differences in adult body weight rather than in numbers or frequency of ovulations or reproductive pattern. The range of species tested did not, however, include those having prodigious ovulation rates, and it will be interesting to compare the numbers of small follicles on a body weight basis in the Plains viscacha, *Lagostomus maximus* (Weir, 1971), and the Elephant shrew, *Elephantulus myurus* (Tripp, 1971), when suitable data become available. With the single exception of the domestic bitch, the great majority of follicles in all species were uniovular and, therefore, allometric relationships for follicles can be read for oocytes. The apparently straightforward scaling of the numbers of follicles or oocytes according to body weight contrasts with the pattern in primate testicles which vary in size and, presumably, therefore in gametogenic capacity, according to reproductive behaviour (Harcourt, Harvey, Larson & Short, 1981).

The allometric relationship between the maximum longevity of a species (L, in years) and body weight was found to be $11{\cdot}8M^{0{\cdot}20}$ (Sacher, 1959), which agrees with the present finding of $9{\cdot}85M^{0{\cdot}25}$ for the adult lifespan. A comparison of allometric exponents indicates that the numbers of follicles at puberty rises more steeply with a unit increase in body weight than with life

expectation. This implies that follicular redundancy increases in larger animals. The same conclusion is indicated by the hyperallometric relationship existing between follicle numbers and adult longevity, for which the exponent is $1{\cdot}592 \pm 0{\cdot}188$ ($r^2 = 0{\cdot}84$) (Fig. 2). The slope of the graph does not strictly match the variation in redundancy within the follicular population since ovulation rates tend to vary inversely with body size. It should be pointed out, furthermore, that the distribution of redundant follicles may be asymmetrical. In the Greater horseshoe bat, ovulations occur only in the right ovary (Harrison Matthews, 1937), whereas it appears that follicles are divided approximately equally within pairs of ovaries.

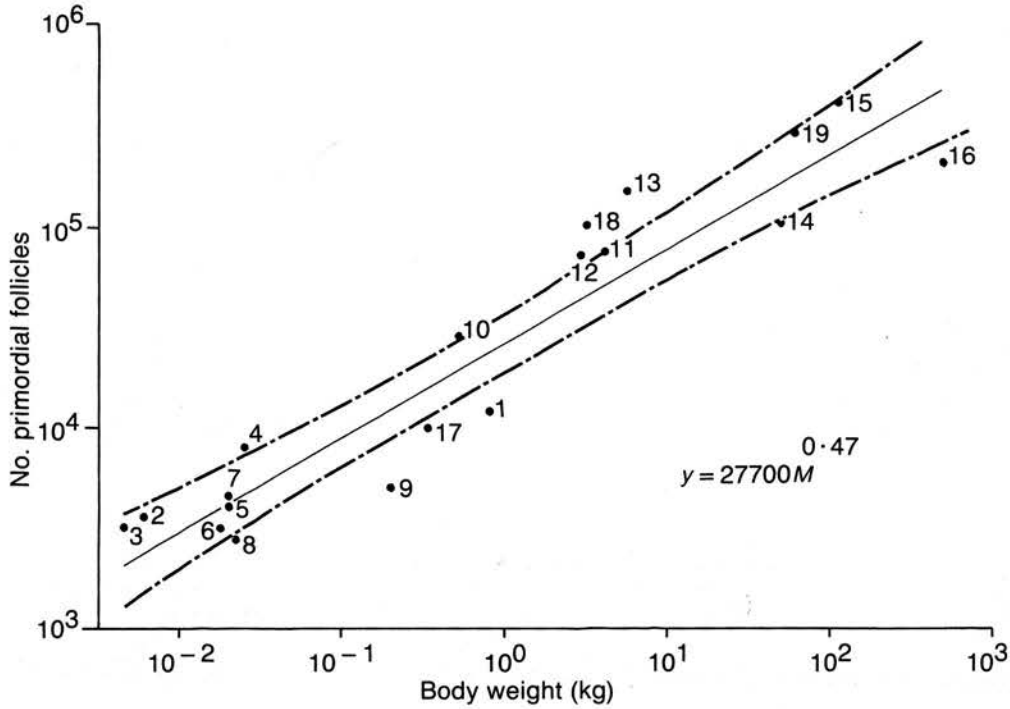

FIG. 1. Variation with body weight in the numbers of primordial follicles per individual at the beginning of adult life. The relationship is depicted by linear regression analysis with the 95% confidence intervals and the allometric formula being shown. See Table I for species code list.

The increased follicular store size in larger species could be an important adaptation for a greater span of adult life. It is presumably due to a greater number of oogonial mitoses during the pre- or perinatal period, although species differences in the rates of germ cell wastage could also contribute to the pattern at puberty. The surplus provision of follicles would appear to provide a wide margin of physiological safety, which has been postulated for other phenotypic characters (Gans, 1979). An alternative theory suggests that a large store reflects the dynamics of postnatal follicle utilization, since follicle numbers are distributed exponentially or multi-exponentially by age, with the store size being substantially reduced or even exhausted at the end

of life (Faddy, Gosden & Edwards, 1983). Accordingly, these dynamics present the ovary with the physiological problem of having to dispose of growing follicles by atresia at young ages when there is a surplus above the small and steady requirements of ovulation. The well-known capacity for superovulation following unilateral ovariectomy or priming with exogenous gonadotrophins may well be an incidental result of a surplus of follicles during the cycle.

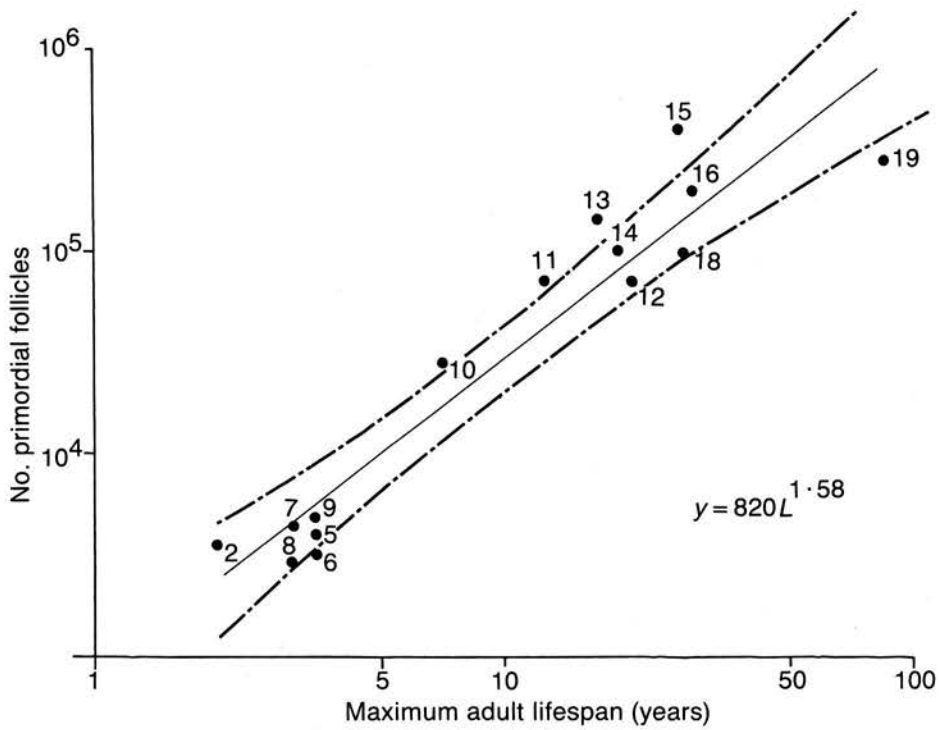

FIG. 2. Variation with the length of the maximum expected adult lifespan in the total numbers of primordial follicles per individual. See also legend to Fig. 1.

A consideration of life history strategies for producing and maintaining germ cell numbers should take account of the rates at which primordial follicles disappear as a result of atresia and recruitment for ovulation. The numbers or fraction of growing follicles are far too variable to be useful indicators of the rate of depletion of the follicle store (Table I). Furthermore, these measurements do not take account of primordial follicles which disappear without having entered the growing follicle population. On the other hand, the slopes of the exponential age distributions of small follicles take account of this potential factor, although data is at present confined to only three species. The slopes obtained for mice, rats and humans are $-1{\cdot}136 \pm 0{\cdot}053$, $-0{\cdot}402 \pm 0{\cdot}025$ and $-0{\cdot}043 \pm 0{\cdot}008$, which correspond to follicle population half-lives of 0·27, 0·75 and 6·99 years, respectively. Thus, the dynamics of follicular utilization would appear to be scaled inversely with body size, indicating that longer-lived species utilize their follicles more conservatively. Data from many more species will be required before any attempts can be

made to test rigorously for allometric variation and, by extension, to determine whether a balance exists between the size of the store and the rate of utilization, so leading to similar residual numbers of follicles near the end of life. Such hypothetical inter-relationships may, however, be difficult to detect because of superpositional effects of the variability of life patterns. For instance, the numbers of follicles were commensurate with body weights in the two species of bats studied (Fig. 1), but they lie far to the right of the distribution with respect to adult longevity based on the limited information which suggests a lifespan of 10–20 years in the natural environment (Fig. 2) (Stebbings, 1977). Compared with rodents and insectivores of similar size, these species may depend on parsimonious utilization of follicles in order to prevent premature loss of fecundity. At present, there are no observations to support this expectation, although the lowering of metabolic rate during hibernation could provide a mechanism.

Primary ovarian failure has rarely been reported to occur before the end of life in animals, except among a few inbred and mutant strains of rodents (Finch & Gosden, 1986). There is, however, the notable exception of menopause in our own species which is usually attained in mid-life and is considered to be primarily a result of follicular deficiency (Gosden, 1985). Since the size of the human follicular population at puberty is consistent with the norms predicted by body weight (Fig. 1), menopause cannot be attributed to an antecedent deficiency. Early ovarian failure in our species could potentially be caused by a comparatively high rate of follicular attrition, but evidence is lacking. There is, however, a *prima facie* case for arguing that menopause has arisen adventitiously during evolution. Menopause may be a by-product of the extension of lifespan beyond expectation based on body size since, in contrast with the maximum longevity of approximately 100 years, allometry predicts a figure of around 30 years for our species.

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REFERENCES

Abercrombie, M. (1946). Estimation of nuclear population from microtome sections. *Anat. Rec.* **94:** 239–247.

Block, E. (1952). Quantitative morphological investigations of the follicular system in women. *Acta Anat.* **14:** 108–123.

Calder III, W. A. (1984). *Size, function and life history*. Cambridge, MA: Harvard University Press.

Erickson, B. H. (1966). Development and senescence of the postnatal bovine ovary. *J. Anim. Sci.* **25:** 800–805.

Erickson, B. H. (1967). Radioresponse of the pre-puberal porcine ovary. *Int. J. Radiat. Biol.* **13:** 57–67.

Faddy, M. J., Gosden, R. G. & Edwards, R. G. (1983). Ovarian follicle dynamics in mice: a comparative study of three inbred strains and an F1 hybrid. *J. Endocr.* **96:** 23–33.

Finch, C. E. & Gosden, R. G. (1986). Animal models for the human menopause. In *Aging, reproduction and the climacteric*: 3–34. Mastroianni, L. Jr & Paulsen, C. A. (Eds). London: Plenum Press.

Gans, C. (1979). Momentarily excessive construction as the basis for protoadaptation. *Evolution, Lawrence, Kans.* **33:** 227–233.

Gosden, R. G. (1985). *Biology of menopause. The causes and consequences of ovarian ageing*. London: Academic Press.

Harcourt, A. H., Harvey, P. H., Larson, S. G. & Short, R. V. (1981). Testis weight, body weight and breeding system in primates. *Nature, Lond.* **293:** 55–57.

Harrison Matthews, L. (1937). The female sexual cycle in the British horseshoe bats, *Rhinolophus ferrum-equinum insulanus* Barrett-Hamilton and *R. hipposideros minutus* Montagu. *Trans. zool. Soc. Lond.* **23:** 224–255.

Jones, E. C. & Krohn, P. L. (1961). The relationships between age, numbers of oocytes and fertility in virgin and multiparous mice. *J. Endocr.* **21:** 469–495.

Mandl, A. M. & Shelton, M. (1959). A quantitative study of oocytes in young and old nulliparous laboratory rats. *J. Endocr.* **18:** 444–450.

Sacher, G. A. (1959). Relation of lifespan to brain weight and body weight in mammals. *Ciba Fdn Coll.* **5:** 115–141.

Stebbings, R. E. (1977). Order Chiroptera, Bats. In *The handbook of British mammals*: 68–128. Corbet, G. B. & Southern, H. N. (Eds). Oxford: Blackwells.

Tripp, H. R. H. (1971). Reproduction in elephant-shrews (Macroscelididae) with special reference to ovulation and implantation. *J. Reprod. Fert.* **26:** 149–159.

Weir, B. J. (1971). The reproductive organs of the female plains viscacha, *Lagostomus maximus*. *J. Reprod. Fert.* **25:** 365–373.

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A quantitative cytological study of polyovular follicles in mammalian ovaries with particular reference to the domestic bitch (*Canis familiaris*)

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Summary. The incidence of polyovular types in the growing follicle population was estimated using quantitative cytology. Of 15 species studied, polyovular follicles were recorded in the following species and in ascending order of abundance: rabbits, rhesus monkeys, humans, cats, dogs. The incidence in bitches was 14% in animals aged 1–2 years but only 5% at 7–11 years old. The frequency of the various types of polyovular preantral follicle varied inversely with the numbers of oocytes per follicle and the probability of finding a follicle with more than 5 oocytes was remote. In young ovaries the frequency was constant in the early stages of growth but decreased in the largest preantral stage. The pattern in ageing ovaries was, by contrast, one of declining frequency such that few if any polyovular types completed development. The ovary of the ageing bitch was also characterized by a higher incidence of degenerating follicles and a much smaller pool of primordial stages. Polyovular follicles were larger than uniovular types at comparable stages which were defined by the number of granulosa cell layers. Their oocytes were smaller but the overall ooplasmic mass was increased with a corresponding increase in the mass of granulosa cells.

Introduction

The majority of ovarian follicles contain only a single oocyte although many authors have drawn attention to instances in which two or more oocytes are present. Binovular and multiovular follicles (henceforward called polyovular follicles) have been reported sporadically in most mammalian species since Von Baer's discovery of the oocyte (Von Baer, 1827). The early literature on polyovular follicles was primarily descriptive histology and was reviewed by Hartman in 1926, but subsequent work has mainly contributed to the range of species in which these are found rather than attempting the larger task of quantitation (see Mossman & Duke, 1973). Progress towards obtaining reliable data and, hence, making inferences about the origin and developmental potential of these structures has undoubtedly been hampered by the problem that polyovular follicles tend to occur infrequently in most species.

Interest in polyovular follicles has stemmed from two questions, namely, their ontogenesis and their contribution to fecundity. These follicles have frequently been regarded as pathological entities and this has led many investigators to pay attention to the incidence of oocyte death and the role of sex steroid and gonadotrophin concentrations (Lane, 1938; Bodemer & Warnick, 1961a; Kent & Mandel, 1970). The changing hormonal 'balance' after puberty provided an explanation for the apparent fall in the incidence of polyovular follicles as animals matured (Kent, 1960). The early inquiries were, however, influenced by a limited knowledge of reproductive endocrinology and the widespread and erroneous belief that oocytes are formed continually throughout life. Polyovular follicles are also of interest because they present a natural experiment in which the general rule of one oocyte:one follicle has been altered with possible developmental significance for the follicle and its various compartments.

The present study was devised to obtain quantitative data about polyovular follicles at different developmental stages in order to identify patterns, to draw inferences about ontogenesis and to make predictions about the contribution of these structures to ovulation at different ages. The data also provided an opportunity to make an initial judgment whether the development of polyovular follicles is substantially different from that of uniovular types.

These objectives depended on the availability of ovaries having a sufficiently high incidence of polyovular follicles. Since published reports rarely present numerical data and where these exist they are not based on a standard classification of follicles, a survey was carried out to identify a suitable species for detailed study. The domestic bitch was chosen on the basis of having a higher frequency of polyovular follicles than did any of the other species studied.

Materials and Methods

Incidence of polyovular follicles in different species. The proportions of growing follicles containing 2, 3, 4 or more oocytes were estimated using histological specimens which had been collected during an earlier study (Gosden & Telfer, 1987). Fifteen species were examined *in toto* (see Table 1) with at least four young adult individuals representing each species except for man for which only one ovary from each of two patients was available. A minimum of 1000 follicles was examined for each species. The nucleolus of the oocyte was used as a marker for counting the follicles and, when appropriate, adjacent sections were searched to determine the complete set of oocytes in polyovular follicles.

On the basis of the findings, additional specimens were obtained from bitches for further studies.

Source and preparation of specimens. Ovaries were obtained during routine veterinary spaying of cross-bred bitches weighing 3·8–21·5 kg. The animals comprised a group of 10 virgins aged 1–2 years old and another group of 5 aged 7–11 years with reproductive histories. All animals were anoestrous and healthy at the time of surgery.

The reproductive tracts were immersed in buffered formalin for transit to the laboratory where the ovaries were dissected and fixed in Susa for 24 h. One ovary from each pair was prepared by serially sectioning at 10 μm and staining with haematoxylin and eosin.

Classification of follicles in bitch ovaries. Each follicle was classified according to the 'stage' in the developmental continuum ('stage') and the 'type' of follicle, as defined by the numbers of oocytes present. The follicle stages were based on the scheme used for rat ovaries by Mandl & Zuckerman (1951) in which the appearance of the oocyte and the number and morphology of the granulosa cell layers are the defining variables (Fig. 1). The follicle types were defined according to the numbers of oocytes present; i.e. type 1 contained a single oocyte, type 2 contained two oocytes, and so on to type 5+. Follicles were also distinguished according to their qualitative appearance: those having a wrinkled oocyte(s) and/or pycnotic granulosa cells were deemed to be degenerating ('atretic'). The data presented in the 'Results' comprise only follicles which appeared to be healthy because it was frequently not possible to assign degenerating follicles to a definite stage and type.

Protocol for counting bitch follicles. A sampling frequency of every 20th serial section was used because a single bitch ovary may yield more than a thousand 10 μm sections. Primordial (non-growing) follicles and uniovular growing stages were counted using the oocyte nucleolus as a marker as this is small and discrete and normally single. The total number of follicles per animal was estimated by multiplying by (a) the sampling frequency (20), (b) a correction factor because of overcounting biasses, and (c) 2 to obtain the value for a pair of ovaries. The correction factor was obtained by a standard method based on the mean diameter of the nucleolus and the section thickness (Mandl & Zuckerman, 1951).

Primordial follicles could not be characterized as polyovular because the boundaries of these small follicles are not easily resolved using the light microscope. The procedure for counting the numbers of polyovular growing follicles differed from that used with the uniovular types. Having identified at least one marker in a polyovular follicle, adjacent sections were searched to determine the total complement of markers (oocytes) present. In general, this added step was important only in larger follicles (stage V) in which the oocytes were more widely dispersed. The product of the raw counts and the sampling frequency produces the estimated numbers of polyovular follicles in one ovary. This method may, however, overestimate the actual number present because follicles carrying a larger number of dispersed oocytes are more likely to be detected in the samples. This problem is not amenable to a simple theoretical approach for obtaining correction factors, as has been the case for uniovular follicles, because there are several markers present and any given section may not contain all of them. Since an examination of every section is impractical, a correction factor was obtained by the following empirical method. The number of polyovular follicles at an intermediate stage (III) was counted in 200 consecutive sections in each of 3 ovaries. The correction factor was obtained by dividing this number by the number obtained from sampling every 20th section. Pilot studies showed that the proportions of follicles that were polyovular differed only marginally after applying the corrected values.

Measurements of follicle and oocyte dimensions. A random sample of 206 uni- and polyovular follicles was used to measure follicles and oocytes in their equatorial planes, as follows. (1) The mean follicular diameter was obtained by

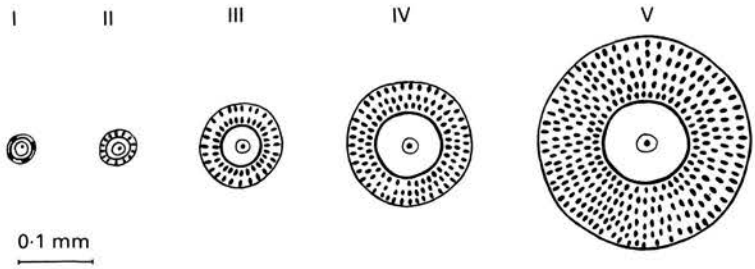

Fig. 1. Schematic representations of preantral stages of follicular growth in bitch ovaries. The figure is drawn to scale and depicts only the uniovular follicle types.

measuring the maximum and minimum diameters using a microscope fitted with an ocular micrometer. The boundary of the follicle was defined by the outer circumference of the granulosa layer because the theca layer was indistinct in small follicles. (2) The diameters of oocytes and their nuclei were measured by a similar method. (3) Precise measurements of nucleolar diameters were obtained using an image-shearing device with oil immersion optics (× 1000) which provide a theoretical resolution of less than 0·2 μm (Swyte & Rosberry, 1977).

Results

Incidence of polyovular follicles in different species

Uniovular follicles were the predominant type in the animals investigated. In 10 of the 15 species studied polyovular follicles were not detected although further and more extensive searches sometimes revealed small numbers of binovular types. The incidence of polyovular follicles in the other 5 species varied from 1% in the rabbit to 14% in the bitch; in human ovaries 3% of the follicles were either bin- or trinovular (Table 1). In each ovary the numbers of follicles of the different types varied inversely with the numbers of oocytes that they contained.

The follicle profile of the anoestrous bitch ovary

The follicles were confined to a narrow cortical band and the large volume of medullary tissue was afollicular and fibrous. Primordial follicles were frequently clustered but the boundaries of these small groups were not clearly circumscribed at the light microscope level (Fig. 2); consequently, groups of oocytes which may be predisposed to polyovular development could not be identified at this stage. Polynuclear oocytes were rarely encountered. Some primordial follicles appeared to be in the process of degeneration but atresia was more clearly characterized in growing follicles in which the oocyte appeared wrinkled and the granulosa cells pycnotic. Polyovular follicles (Fig. 3) could not be confused with atretic follicles containing a fragmented oocyte since a distinct nucleus was present in each of the oocytes. About 10% of the growing follicle population in young ovaries was atretic by these criteria, as opposed to 30% in the older bitches. The proportions of the different types of polyovular development were remarkably consistent from stage to stage. Anovular 'follicles' were observed occasionally in ageing ovaries; the space formerly occupied by the oocyte had been invaded by granulosa cells.

The total numbers of primordial follicles per animal were highly variable (Table 2). The mean in young ovaries was 85 800 (s.e. 18 600) compared with only 2750 (s.e. 900) in the older group ($P < 0{\cdot}05$ using the Kruskal Wallis non-parametric test). The primordial follicle was the most abundant stage present and, like the other stages, suffered substantial losses during ageing. In contrast to these reductions, the percentage of follicles in the growing stages rose substantially since the relative age-dependent losses in the growing and non-growing sub-populations differed.

Table 1. Frequency of polyovular follicles in 15 mammalian species

Species		% of growing follicles containing:		
Common name	Specific name	2 oocytes	3 oocytes	4+ oocytes
Bandicoot	*Isoodon macrourus*	<0·10	<0·10	<0·10
Common shrew	*Sorex araneus*	<0·10	<0·10	<0·10
Pipistrelle bat	*Pipistrellus pipistrellus*	<0·10	<0·10	<0·10
Laboratory mouse (CBA)	*Mus musculus*	<0·10	<0·10	<0·10
Woodmouse	*Apodemus sylvaticus*	<0·10	<0·10	<0·10
Bank vole	*Clethrionomys glareolus*	<0·10	<0·10	<0·10
Field vole	*Microtus agrestis*	<0·10	<0·10	<0·10
Rat	*Rattus norvegicus*	<0·10	<0·10	<0·10
Rabbit	*Oryctolagus cuniculus*	0·91	<0·10	<0·10
Cat	*Felis catus*	3·61	0·45	<0·10
Bitch	*Canis familiaris*	8·89	2·97	2·08
Sheep	*Ovis aries*	<0·10	<0·10	<0·10
Marmoset monkey	*Callithrix jacchus*	<0·10	<0·10	<0·10
Rhesus monkey	*Macaca mulatta*	1·49	0·30	<0·10
Human	*Homo sapiens*	2·72	0·19	<0·10

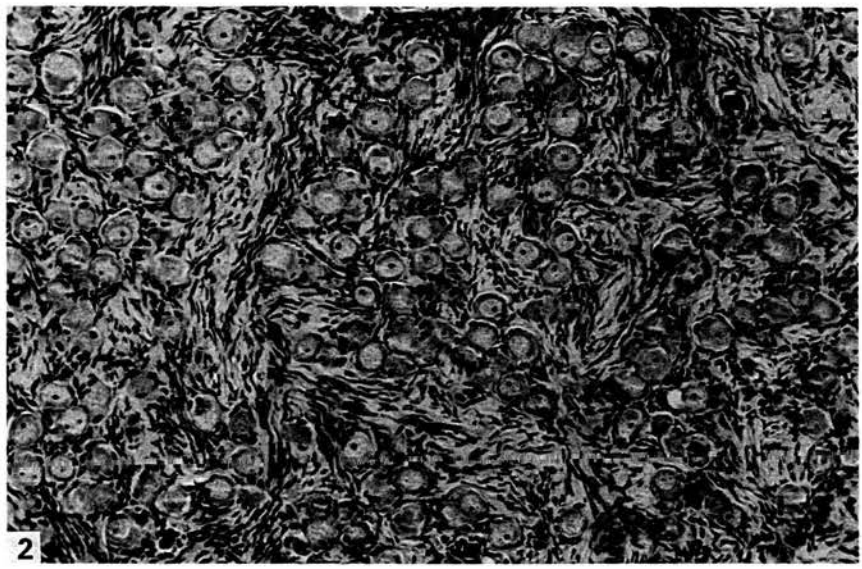

Fig. 2. The ovarian cortex of a young adult bitch which contains numerous primordial follicles in clusters. H & E, × 160.

Whereas in young animals only 10% of follicles were classified as growing, over 50% were growing in the older group.

Polyovular follicles were present in all animals with the single exception of a young bitch which, except when stated otherwise, has been included in the presentation of data. They were found at each of the four stages of preantral growth (II–V) in young ovaries and at stages II–IV in the other groups (Tables 3 & 5). Overall, their incidence was similar to that obtained during the pilot studies using a small sample (Table 1), being 14% in the young group but only 5% in the old animals.

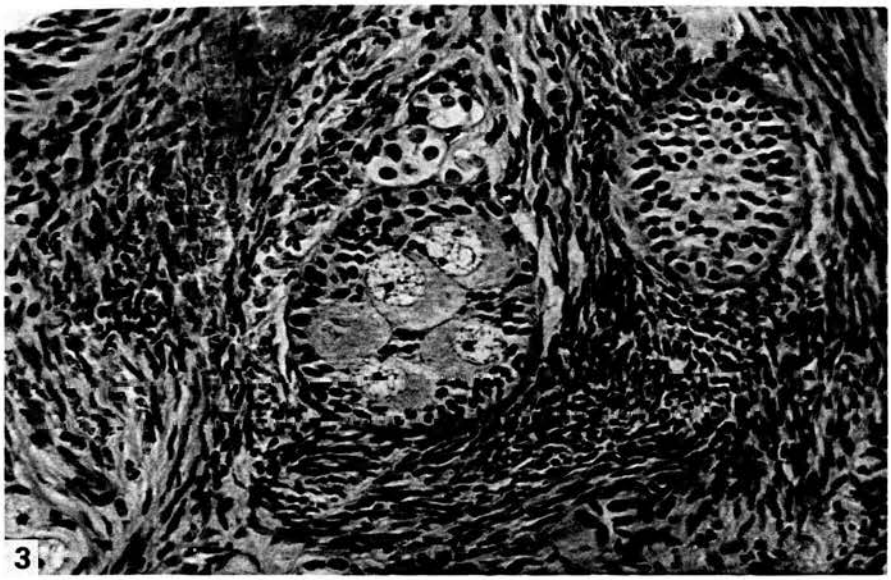

Fig. 3. Detail of the cortex of the bitch ovary showing a growing follicle containing 5 oocytes (centre). H & E, ×320.

Table 2. A quantitative profile of the follicle population of anoestrous ovaries in young adult and ageing bitches

	Young animals	Ageing animals
No. of animals	10	5
Age range (years)	1–2	7–11
No. of primordial follicles (range)	5400–214 000	1200–6150
No. of growing follicles (range)	3470–13 600	1720–6770
% growing follicles	10	51
No. of polyovular growing follicles (range)	423–5930†	62–238
% polyovular growing follicles	14	5

†Excludes one animal in which polyovular follicles were absent.

Table 3. Numbers (and s.e.m.) of growing follicles in young bitch ovaries classified according to the stage of development and number of oocytes ('type')

Follicle stage	Follicle type				
	1	2	3	4	5+
II	4002 (585)	378 (104)	119 (47)	56 (34)	49 (34)
III	2089 (32)	210 (77)	90 (43)	40 (57)	27 (20)
IV	972 (132)	147 (60)	59 (30)	20 (13)	5 (5)
V	1251 (209)	128 (60)	13 (14)	4 (3)	2 (2)

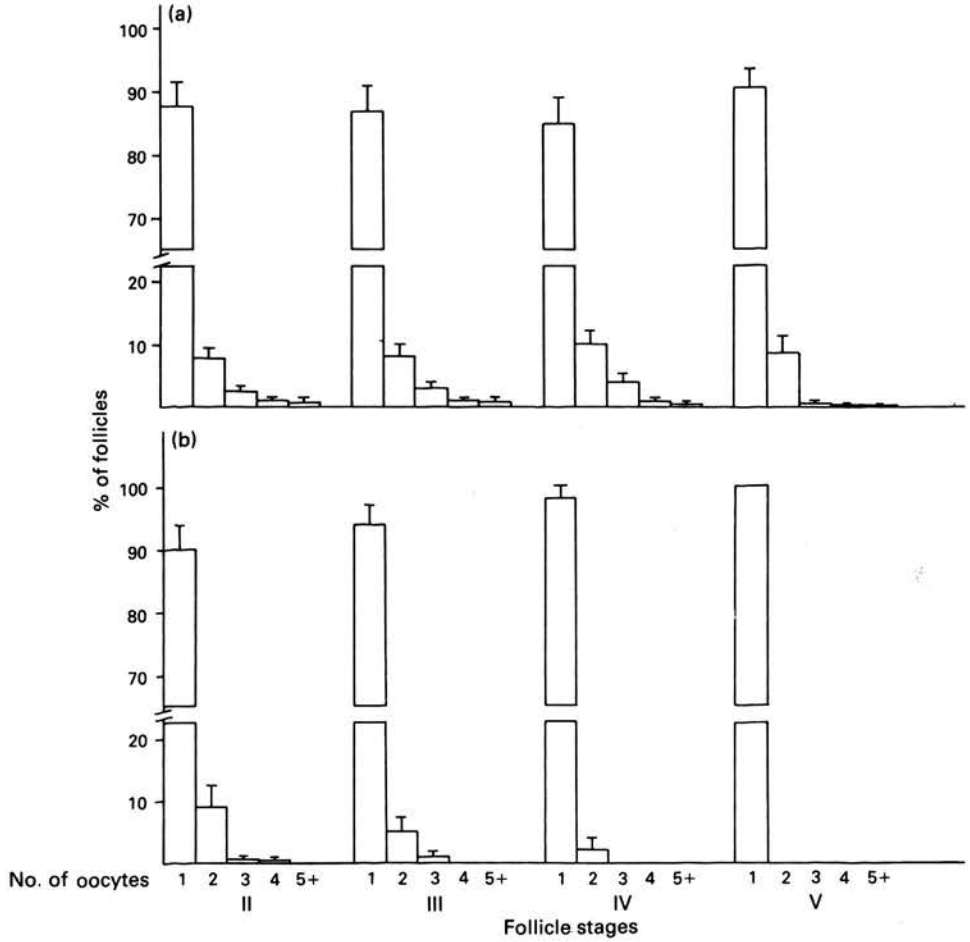

Fig. 4. Percentage frequency distribution at successive stages of development of polyovular follicles in ovaries of (a) young bitches (N = 10) and (b) ageing bitches (N = 5). Values given are mean + s.e.m.

The consistent proportions of polyovular follicles from stage to stage were striking in view of the variable numbers of follicles present (Fig. 4a). In young animals, it was only at stage V that the proportions of these follicles fell and this effect was restricted to types 3+ ($P < 0{\cdot}01$). The numbers at any given stage appeared to decline approximately exponentially as the numbers of oocytes rose. Since estimating the incidence of follicles with more than 5 oocytes was hazardous because of sampling errors at low frequencies the tabulated results and histograms have not been extended beyond type 5+ in which the less common types were combined. The largest number of oocytes observed in a single follicle was 9 in this series although up to 14 were observed in other bitches. A simple probability table was constructed to indicate the chances of encountering growing follicles of a given type and stage (Table 4). This demonstrates that the probability declines steadily from left to right as the numbers of oocytes increase, in contrast there is less variation on the vertical scale which represents successive stages of follicle growth. Clearly, a large number of follicles must be sampled to obtain reliable estimates of follicles with 4 or more oocytes.

The incidence of different follicle types in older bitches demonstrated a pattern similar to that described in the younger group (Fig. 4b) although there were two major differences between them.

Table 4. Probability distribution for follicles in young bitch ovaries classified according to developmental stage and number of oocytes

Follicle stage	Follicle type				
	1	2	3	4	5+
II	0·414	0·040	0·013	0·006	0·005
III	0·216	0·022	0·009	0·004	0·003
IV	0·100	0·015	0·006	0·002	<0·001
V	0·129	0·013	0·001	<0·001	<0·001

Table 5. Numbers (and s.e.m.) of growing follicles in ageing bitch ovaries classified according to the stage of development and number of oocytes

Follicle stage	Follicle type				
	1	2	3	4	5+
II	1308 (387)	100 (38)	8 (4)	4 (4)	0
III	828 (270)	30 (15)	4 (4)	0	0
IV	343 (174)	4 (4)	0	0	0
V	364 (167)	0	0	0	0

Table 6. Probability distribution for follicles in ageing bitch ovaries classified according to developmental stage and numbers of oocytes

Follicle stage	Follicle type				
	1	2	3	4	5+
II	0·440	0·033	0·003	0·001	<0·001
III	0·276	0·010	0·001	<0·001	<0·001
IV	0·114	0·001	<0·001	<0·001	<0·001
V	0·122	<0·001	<0·001	<0·001	<0·001

Firstly, there was the overall reduction in numbers and incidence of polyovuly as was mentioned above. Secondly, the consistent proportions of polyovular follicles from stage to stage was lost. Despite having sampled hundreds of sections of ageing ovaries polyovular follicles were never detected at stage V and only a few binovular types were represented at the preceding stage (Table 5). The probability table suggests that the chance of finding a binovular follicle at the earliest stage of growth in older animals is only slightly lower than that for young ovaries whereas that of finding other types rapidly approaches zero (Table 6).

Dimensions of follicles and oocytes

The data were aggregated from young and old animals since the size of follicles and oocytes were independent of age. The diameter of oocytes increased in direct proportion to that of the follicle until the latter had reached approximately 300 μm at stage V when the oocyte had attained

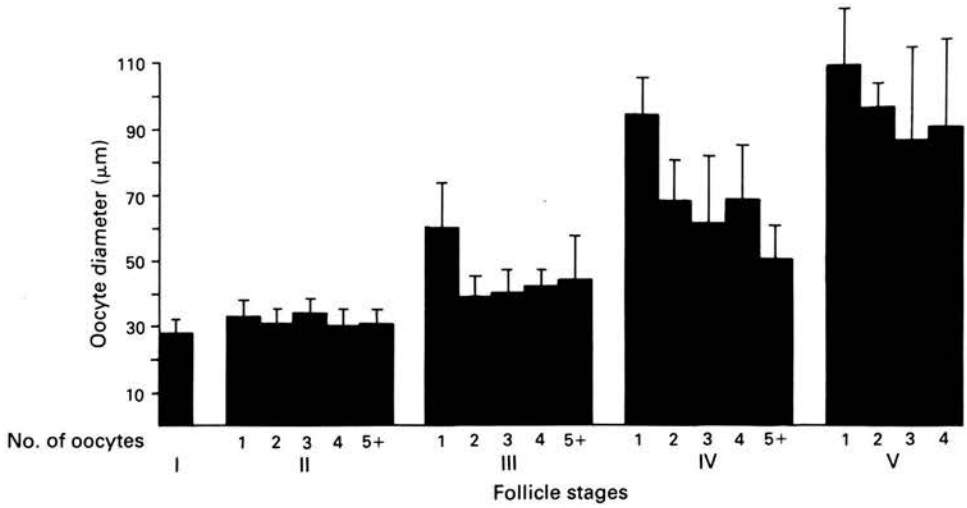

Fig. 5. Variation in the diameters of oocytes in uni- and polyovular follicles at different stages of development in bitch ovaries. Values are means + s.e.m.

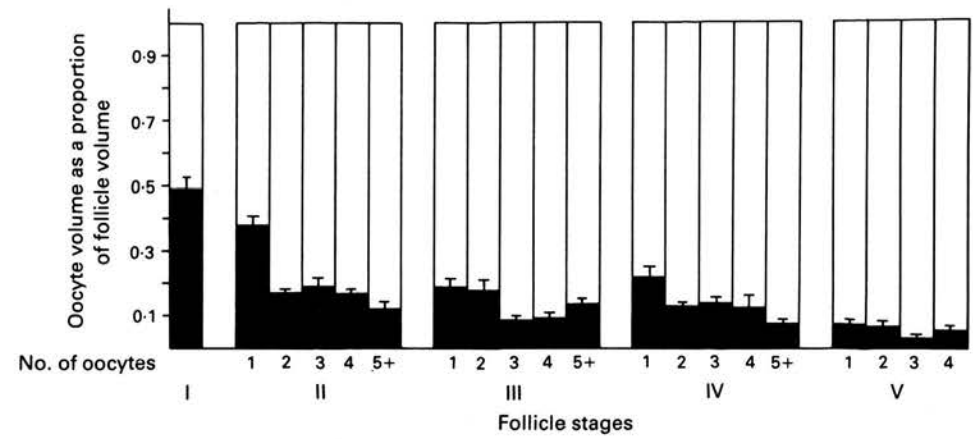

Fig. 6. The proportion of follicle volume occupied by the oocyte(s): variation with the stage of development and the numbers of oocytes per follicle. Values are mean and s.d. because the s.e. values were too small to be drawn.

its mature size of 90–110 μm. Whilst the size of oocytes was similar in polyovular follicles at stage II, at subsequent stages it was always greater in uniovular types (Fig. 5). Frequently, the oocytes were packed closely to one another near the geometric centre of the follicle (Fig. 3) but when they were more scattered it appeared that the peripheral oocytes were smaller than the central ones.

Polyovular follicles were substantially larger than uniovular types at the same stage of development. This difference, which was 2–3-fold in volume, could be accounted for by both a greater volume of oocytes *in toto* and larger numbers of granulosa cells. The granulosa cell volume was deduced from the difference between the follicle volume and the total volume of oocyte(s) on the assumption that the volume of individual granulosa cells is constant and that there is little extracellular space at preantral stages. Despite the smaller sizes of individual oocytes the total volume in polyovular follicles was increased. The relationship between the volume of the oocyte(s)

and that of the follicle was not constant and when expressed as proportions there was a tendency for the granulosa cell component to rise during development (Fig. 6). The proportion occupied by oocytes was greatest at stage II for uniovular follicles, this being significantly greater than in comparable polyovular types ($P < 0{\cdot}05$). At later stages the proportions were relatively even between types.

Discussion

The quantitative profile of follicles in the bitch ovary conveys the impression that polyovular development is neither a spurious nor a pathological phenomenon but is a natural polymorphism arising from a spectrum of possible numerical combinations of oocytes and pregranulosa cells. Uniovular development is the dominant mode in all species investigated but polyovuly is widely distributed albeit at varying frequencies (see also Bocharov, 1966; Mossman & Duke, 1973).

These new data are consistent with, but do not prove, the hypothesis that polyovular follicles are predetermined during folliculogenesis. Concrescence was not observed between growing follicles and this possibility is apparently denied by the stable or falling incidence of polyovuly at successive stages. A third explanation for the presence of polyovular follicles proposes that either polynucleate or mononucleate oocytes divide (Hartman, 1926; Iguchi *et al.*, 1986) but this can be refuted in the present examples because of a deficiency of the former with a lack of excess numbers of follicles with an even number of oocytes. Whilst there is circumstantial evidence for the predetermination of polyovular follicles the latter cannot be claimed to pre-exist in the primordial follicle pool since there is neither cytological nor physiological evidence of intercellular metabolic coupling. Light microscopy gives only a suggestion of physical intimacy between oocytes but fine structural details must be clarified if the possibility of synchronous development is to be inferred. Cortical oocytes occur in dense plaques in young ovaries of species having abundant polyovular follicles, but density-dependence cannot be the only causal factor since intimately clustered oocytes occur in some species which rarely present polyovuly (e.g. sheep).

One of the striking features of the data is the inverse relationship between the numbers of oocytes per follicle and the numbers of follicles. The frequency distribution suggests that a stochastic process has been operating although this hypothesis cannot be verified in the adult ovary since utilization and death of follicles has modified the original population and, additionally, incipient polyovular follicles cannot be identified. The conclusion that polyovuly arises from random events during folliculogenesis conflicts with most of the suggestions in the older literature. On the basis of early claims that polyovular follicles were less common after puberty than in the immature ovary (Davis & Hall, 1950; Dawson, 1951; Bodemer & Warnick, 1961b) some workers obtained evidence that the incidence of these follicles depends upon the balance of sex steroids and pituitary gonadotrophins (Kent, 1959; Graham & Bradley, 1971). The present results do not deny a possible role for these hormones, but the predetermination hypothesis proposes an explanation for polyovuly on the basis of cellular interactions occurring during folliculogenesis which may or may not depend on sex steroids and the gonadotrophins.

Polyovular follicles are less common in older bitches than in animals beginning their reproductive life: they are fewer in number and comprise a lower percentage of the growing follicle population. As more specimens become available it will be interesting to determine whether polyovular follicles become progressively more sparse throughout life. Whilst the probability of finding a binovular follicle was only slightly reduced during ageing, those for types 3 and 4 were considerably lower and other types were entirely absent. Since the animals were anoestrous it is unclear whether there is a hormonal basis for these findings. The age effect could be due to a reduced frequency of predetermined polyovular follicles in the reserve pool as a result of increased utilization at young ages and/or decreased viability of follicles entering their growth phase. The nature of the stimulus that prompts primordial follicles to start growing is not known, although it is

widely assumed to be a spontaneous event. If it is conjectured that this event occurs independently in oocytes and that activation can be transmitted to physiologically coupled oocytes it is to be expected that the probability of utilization will vary with the number of oocytes within a follicle. This model is in accord with the observed changes in the follicle profile. On the other hand, the diminishing frequency of polyovular follicles at each successive stage of growth suggests that they are less viable in old ovaries. The environment of the ageing ovary may be less capable of providing physiological support for the growth of follicles, and this is consistent with the observed higher incidence of follicle death.

Oocytes in polyovular follicles start their development at a size similar to that of oocytes in uniovular follicles. Size differences emerge, however, at stage III when two granulosa cell layers are present but the oocytes undergo morphologically normal development. This may suggest some developmental lagging but it could also be argued that the stages of uni- and polyovular follicles are not comparable. This hypothesis is difficult to evaluate from a morphological approach.

Polyovular follicles were larger than uniovular types at the same stage and it will be interesting to determine whether these differences continue into the antral stages. The number of granulosa cells inferred from measurements of follicle and oocyte volumes rose *pari passu* with the total mass of oocyte(s), which may indicate that cellular interactions are co-ordinating the growth of the compartments. There would appear from the morphological evidence to be no reason to suspect that oocytes in young ovaries are much less viable in polyovular follicles compared with the norm and other studies have shown that they can reach preovulatory maturity, each being enclosed in separate cumuli oophori (Dederer, 1934; Davis & Hall, 1950; Andersen & Simpson, 1973).

In addition to the problem of ontogenesis, polyovular follicles have attracted interest because of their potential contribution to the quota of oocytes shed at ovulation and, hence, to the species-characteristic litter size. According to the present results this contribution is expected to be small even in species having relatively abundant polyovular follicles and it is likely to be virtually nil in older animals. The quantitative significance of these follicles can be illustrated by a simplified example. If it is assumed that all oocytes in stage V follicles are equally viable and that the selection of dominant follicles is random, the chance of ovulating a polyovular follicle of any type would be a product of their incidence at that stage and the number of follicles selected for ovulation. We can see from the data that the pool of follicles available for ovulation contains 10 times as many uniovular follicles as polyovular follicles. Assuming that the mean ovulation rate in the bitch is four and using the values obtained for follicle type at stage V, we can estimate that the probability of one binovular follicle being ovulated will be 0·06. Because estimates based on the mixed group of follicles at stage V do not represent the ovulatory pool of follicles, any probability values are likely to be overestimates. In view of these predictions and the observed age change it appears that the rising fecundity during the first half of the reproductive lifespan, as measured by the frequency of dizygotic twins in women (Bulmer, 1970) and the successively larger litters in young parous mice (Kennedy & Kennedy, 1972), is probably due to a higher ovulation rate rather than the contribution of polyovular follicles.

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References

Andersen, A.C. & Simpson, M.E. (1973) *The Ovary and the Reproductive Cycle of the Dog (Beagle)*. Geron-X Inc., Los Altos, California.

Bocharov, W.S. (1966) Multiovular follicles in the ovaries of mammals. *Adv. Sov. Biol.* **62,** 139–147.

Bodemer, C.W. & Warnick, S. (1961a) Polyovular follicles in the immature hamster ovary. II. The effects of gonadotropic hormones on polyovular follicles. *Fert. Steril.* **12,** 353–364.

Bodemer, C.W. & Warnick, S. (1961b) Polyovular fol-

licles in the immature hamster ovary. I. Polyovular follicles in the normal intact animal. *Fert. Steril.* **12,** 159–169.

Bulmer, M.G. (1970) *The Biology of Twinning in Man.* Oxford University Press, London.

Davis, D.E. & Hall, O. (1950) Polyovuly and anovular follicles in the wild Norway rat. *Anat. Rec.* **107,** 187–192.

Dawson, A.B. (1951) Histogenetic interrelationships of oocytes and follicle cells. A possible explanation of the mode of origin of certain polyovular follicles in the immature rat. *Anat. Rec.* **110,** 181–197.

Dederer, P.H. (1934) Polyovular follicles in the cat. *Anat. Rec.* **60,** 391–396.

Gosden, R.G. & Telfer E. (1987) Numbers of follicles and oocytes in mammalian ovaries and their allometric relationships. *J. Zool., Lond.* **211,** 169–175.

Graham, C.E. & Bradley, C.F. (1971) Polyovular follicles in squirrel monkeys after prolonged diethylstilboestrol treatment. *J. Reprod. Fert.* **27,** 181–185.

Hartman, C.G. (1926) Polynuclear ova and polyovular follicles in the opossum and other mammals with the special reference to the problem of fecundity. *Am. J. Anat.* **37,** 1–52.

Iguchi, T., Takasugi, N., Bern, H.A. & Mills, K.T. (1986) Frequent occurrence of polyovular follicles in ovaries of mice exposed neonatally to diethyl stilbestrol. *Teratology* **34,** 29–35.

Kennedy, T.G. & Kennedy, J.P. (1972) Effects of age and parity on reproduction in young female mice. *J. Reprod. Fert.* **28,** 77–84.

Kent, H.A., Jr (1959) Reduction of polyovular follicles and polynuclear ova by oestradiol monobenzoate. *Anat. Rec.* **134,** 455–462.

Kent, H.A., Jr (1960) Polyovular follicles and multinuclear ova in ovaries of young mice. *Anat. Rec.* **137,** 521–524.

Kent, H.A., Jr & Mandel, J.A. (1970) Correlation of ovarian abnormalities in young golden hamsters with ovarian oestrogen and progesterone. *Anat. Rec.* **168,** 233–236.

Lane, C.E. (1938) Aberrant ovarian follicles in the immature rat. *Anat. Rec.* **71,** 243–247.

Mandl, A.M. & Zuckerman, S. (1951) Numbers of normal and atretic oocytes in unilaterally spayed rats. *J. Endocr.* **7,** 112–119.

Mossman, H.W. & Duke, K.L. (1973) *Comparative Morphology of the Mammalian Ovary.* University of Wisconsin, Madison, Wisconsin.

Swyte, D.A. & Rosberry, F.W. (1977) A comparison of some optical microscope measurements of photomask linewidths. *Solid State Technol. August,* 70–75.

Von Baer, K.E. (1827) *De ovi mammalium et hominis genesi.* Epistola ad Acad. Imper. Sci. petropolitanam. L. Vossi Leipzig.

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Scaling of follicular sizes in mammalian ovaries

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(With 6 figures in the text)

The scaling of ovarian follicle and oocyte sizes according to body weight (M, ranging from 0·005–500 kg) has been analysed using data obtained from 22 mammalian species in nine orders. The diameters of non-growing (primordial) follicles were correlated significantly with body weight, the relationship being described by the allometric formula $y = 0{\cdot}028M^{0{\cdot}10}$. The mean size at which growing follicles began to accumulate extracellular fluid was approximately the same in all species, 0·3 mm diameter. Graafian follicle sizes varied allometrically with body weight as a result of differences in the volumes of follicular fluid rather than those of oocytes, which were relatively similar in eutherian mammals. The statistical significance of the correlation between Graafian and body sizes was increased when the dimensions for an ovulatory quota of follicles were combined because follicles in polyovulating species were disproportionately small. The total Graafian surface areas and volumes were then predicted from body weight by $58{\cdot}4M^{0{\cdot}65}$ and $18{\cdot}5M^{1{\cdot}06}$, respectively. Follicular dimensions in the three species of primates were significantly greater than predicted by the allometric relationship. The exponents of these relationships show that the total volume of a set of preovulatory follicles varies approximately isometrically with body weight and, therefore, with the presumptive hormone distribution volume ($M^{1{\cdot}0}$). The hypoallometric relationship of follicular surface area demonstrates that, during the course of the evolution of body size, the surface area for secretion has not increased to match the dilution of hormones in the body pool.

Contents

Introduction

A number of variables that characterize patterns of reproductive physiology are scaled allometrically with physical size, evidently contributing to the optimization of reproductive success. Such relationships have been well documented in the cases of variation in litter size, age at puberty, and a number of other variables in mammalian reproduction (May & Rubenstein, 1984), but scaling of the sizes of the reproductive organs has received scant attention.

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TABLE I

List of species investigated and code numbers used in Figures

Code no.	Common name	Specific name	Order
1	Bandicoot	*Isoodon macrourus*	Marsupialia
2	Common shrew	*Sorex araneus*	Insectivora
3	Hedgehog	*Erinaceus europaeus*	,,
4	Elephant shrew	*Elephantulus myurus*	,,
5	Pipistrelle bat	*Pipistrellus pipistrellus*	Chiroptera
6	House mouse	*Mus musculus*	Rodentia
7	Wood mouse	*Apodemus sylvaticus*	,,
8	Bank vole	*Clethrionomys glareolus*	,,
9	Field vole	*Microtus agrestis*	,,
10	Norway rat	*Rattus norvegicus*	,,
11	Guinea pig	*Cavia porcellus*	,,
12	Plains viscacha	*Lagostomus maximus*	,,
13	Laboratory rabbit	*Oryctolagus cuniculus*	Lagomorpha
14	Domestic cat	*Felis catus*	Carnivora
15	Domestic dog	*Canis familiaris*	,,
16	Sheep	*Ovis aries*	Artiodactyla
17	Swine	*Sus scrofa*	,,
18	Cattle	*Bos taurus*	,,
19	Horse	*Equus caballus*	Perissodactyla
20	Common marmoset	*Callithrix jacchus*	Primates
21	Rhesus monkey	*Macaca mulatta*	,,
22	Human	*Homo sapiens*	,,

In primates, testicular weights, which are indicative of spermatogenic capacity, present allometric variation, but superimposed upon this are the effects of sperm competition in some species (Harcourt, Harvey, Larson & Short, 1981). A scaling relationship exists in the ovary between the size of the store of non-growing (primordial) follicles and body weight (Gosden & Telfer, 1987), but the sum of these units is only a small proportion of the organ size overall. Most of the volume of young adult ovaries derives from Graafian follicles or corpora lutea, depending on the phase of the ovarian cycle.

Parkes (1932) reported that Graafian follicle diameters vary linearly with body weight when the axes are transformed logarithmically, implying an allometric relationship, but this conclusion was based on only seven species. His finding is verified in the present paper in which the study has been extended to other follicle stages using a larger set of data. Allometric expressions have been obtained to describe relationships, and these may guide theory about the underlying physiological bases of follicle size and scale.

Materials and methods

Sources of ovarian data

Data were obtained from young, adult nulliparous individuals either at autopsy or at ovariectomy. The species are listed in Table I. Measurements of Graafian follicles were taken shortly before the expected time of ovulation, whereas those of smaller stages were not so constrained. The ovaries were fixed, embedded in paraffin wax, sectioned serially at 7 or 8 μm and stained with haematoxylin and eosin.

Mensuration of ovarian follicles and oocytes

The mean diameters of non-atretic follicles and of oocytes in Graafian follicles were obtained in the equatorial tissue sections from the average of the maximum diameter and the diameter at right angles. They were based on 8–30 measurements from 4–8 individuals in each species. Follicles were measured at 3 distinctive stages of development: (1) primordial follicles in which the epithelium was unilaminar and squamous and a small oocyte was present; (2) solid, multilaminar follicles in which formation of pools of follicular fluid was incipient; (3) mature Graafian follicles shortly before ovulation. The larger Graafian dimensions among species exceeding 1 kg body weight were obtained by measuring fresh materials with calipers. In the carnivores, primates and some artiodactyls, the data were collected from published studies. The surface areas and volumes of Graafian follicles have been estimated because these variables could be physiologically more significant than the follicular diameter (D). The estimates were based on the assumption that follicles are spherical with surface areas and volumes corresponding to $4\pi(D/2)^2$ and $1{\cdot}33\pi(D/2)^3$, respectively. The morphological appearance of follicles upheld this assumption in all cases except the domestic bitch, in which the surface area presented for diffusion to and from the vascular theca will have been underestimated because of the natural folding of the follicular wall.

Body weight and numbers of primordial and ovulatory follicles

The body weights and the numbers of ovulations per cycle (ovulation rates) were obtained for young adult individuals during the course of the study, or were obtained from published sources (e.g. Altman & Dittmer, 1972). The ovulation rate has been taken to indicate the numbers of preovulatory follicles when estimating dimensions collectively. The mean numbers of primordial follicles in pairs of ovaries were obtained during a concurrent study which was based mainly on the same specimens (Gosden & Telfer, 1987).

Statistics

The mean values for each of the variables were used for analysing interspecific patterns. They were transformed to common logarithms for graphical representation and for testing whether relationships were consistent with the allometric formula, $y = aM^b$. Linear regression analysis was carried out by the method of least squares and the 95% confidence limits were calculated.

Results

A correlation matrix for the major variables is given in Table II and selected allometric relationships are depicted with their formulae in Figs 2–6.

The diameter of oocytes in Graafian follicles was correlated with body weight at the 5% level of statistical significance ($0{\cdot}05 > P > 0{\cdot}02$); a regression line has not therefore been fitted (Fig. 1). The diameter of oocytes in Graafian follicles varied among eutherian mammals from 0·058 mm in small rodents to 0·113 mm in sheep; in the marsupial species it was 0·133 mm.

Diameters of primordial follicles ranged from 0·014–0·093 mm. When plotted against body weight on logarithmic scales, a linear correlation was obtained ($P < 0{\cdot}01$) in which the coefficient of determination (r^2) was 0·45 (Fig. 2). The interspecific differences in sizes of these follicles were mainly due to the volumes of ooplasm, which were disproportionately large in relation to body size in rabbits and cats. These sizes showed statistically significant correlations with both the numbers present at young adult ages and with the sizes of Graafian follicles and of oocytes ($P < 0{\cdot}01$) (Table II).

TABLE II

Correlation matrix for logarithmically transformed values for body weight and ovarian parameters

		Body wt.	Number of primordial follicles	Ovulation rate (*n*)	Diameter: Ovum	Diameter: Primordial follicle	Diameter: Graafian follicle (GF)	Surface area: GF × 1	Surface area: GF × *n*	Volume: GF × 1
Number of Primordial follicles		0·953								
Ovulation rate (*n*)		−0·270	−0·462							
Diameter	ovum	0·552	0·730	−0·422						
	Primordial follicle	0·669	0·676	−0·308	0·769					
	Graafian follicle (GF)	0·880	0·902	−0·673	0·687	0·575				
Surface area	GF × 1	0·868	0·905	−0·684	0·691	0·561	0·990			
	GF × *n*	0·921	0·930	−0·475	0·686	0·569	0·958	0·967		
Volume	GF × 1	0·869	0·905	−0·684	0·691	0·561	0·990	1·000	0·967	
	GF × *n*	0·921	0·928	−0·558	0·694	0·571	0·978	0·987	0·995	0·987

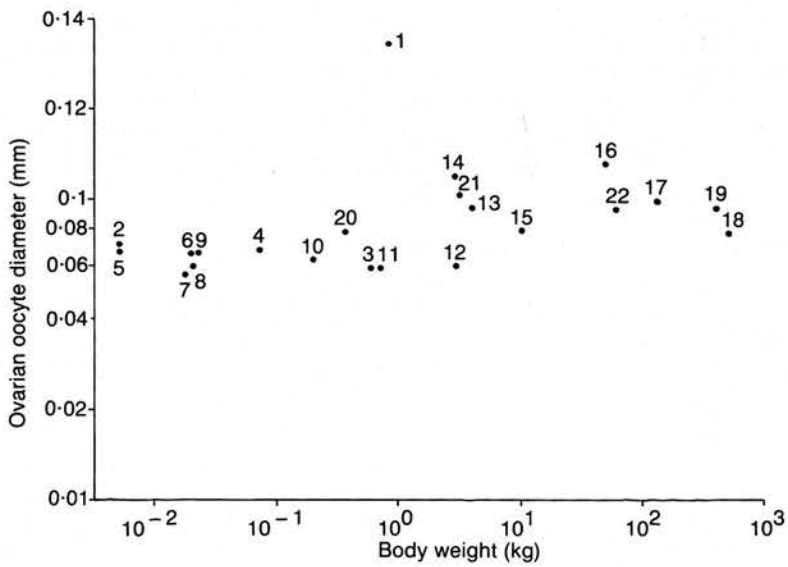

FIG. 1. Variation between mean diameters of oocytes in Graafian follicles and body weight (see Table I for species code list for Figs 1–6).

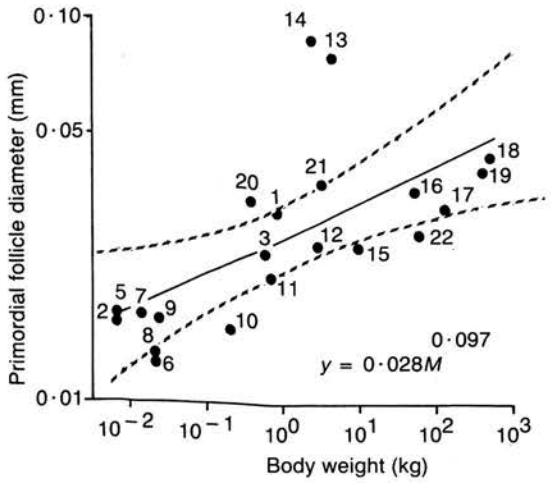

FIG. 2. Variation between mean diameters of primordial follicles and body weight. Here, and in the following Figs, the line of best fit obtained by the least squares method is presented with the 95% confidence interval and allometric formula (see Table I for species code list for Figs 1-6).

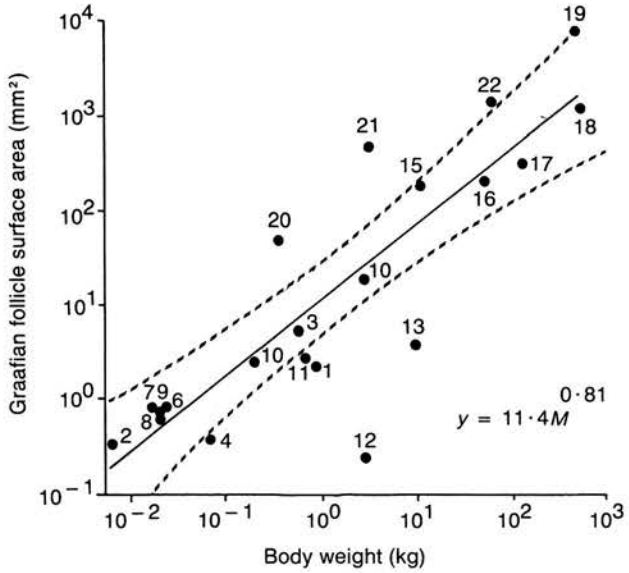

FIG. 3. Allometric relationship between the surface area of individual Graafian follicles and body weight (see Table I for species code list for Figs 1-6).

Mature follicles were characterized by the presence of a follicular antrum in all species examined. The volume of follicular fluid contributed more than 50% to the total volume of mature follicles exceeding 0·4 mm diameter, the percentage being greater in the larger follicles. The epithelium of mural granulosa cells was 3-9 cell layers thick, without any systematic variation

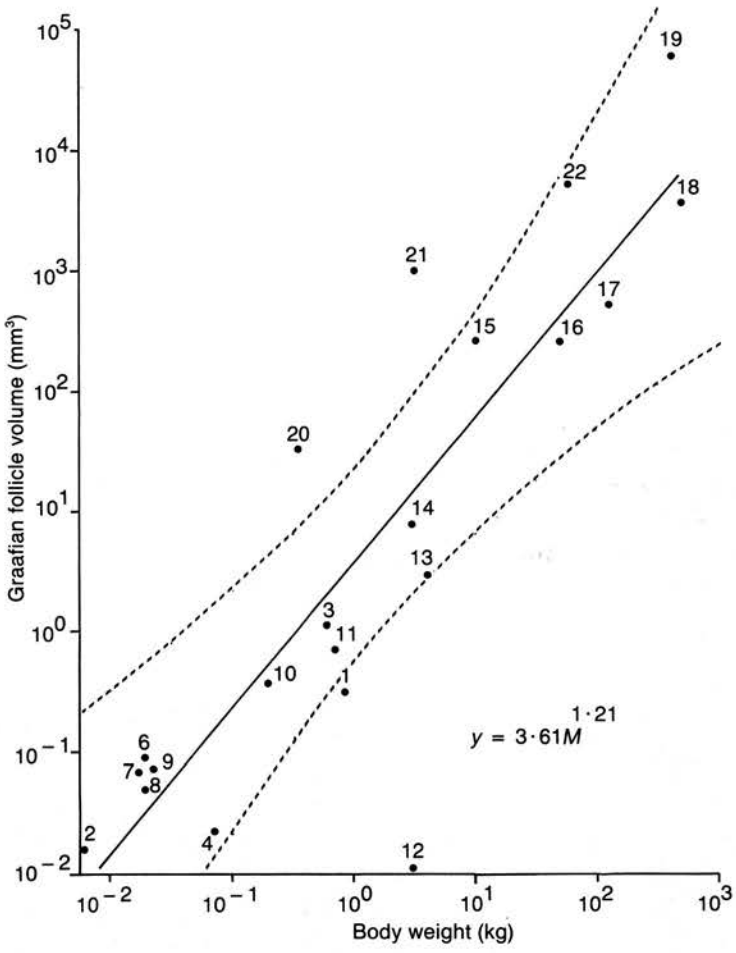

FIG. 4. Allometric relationship between the volume of individual Graafian follicles and body weight (see Table I for species code list for Figs 1–6).

according to follicular diameter. In contrast, the theca layer appeared to consist of more cell layers in larger follicles, but indistinct boundaries prohibited accurate measurement of the theca or of its subdivision: theca interna and externa. The sizes at which follicles were observed to accumulate fluid ranged from 0·2–0·4 mm in diameter in the different species. Considerable variation existed even within a single ovary at this stage, and the transition did not appear to be related to body size or the size of follicles at maturity.

Graafian dimensions ranged from 2–5 orders of magnitude above the minimum values observed in the shrew and Plains viscacha, depending on whether the diameter, surface area or volume was being compared (Figs 3–6). These dimensions were correlated significantly with the diameters of both oocytes and primordial follicles, although only about half or a third of the total variation was so accounted for (r^2 = 0·47 and 0·33, respectively). Differences between species in body

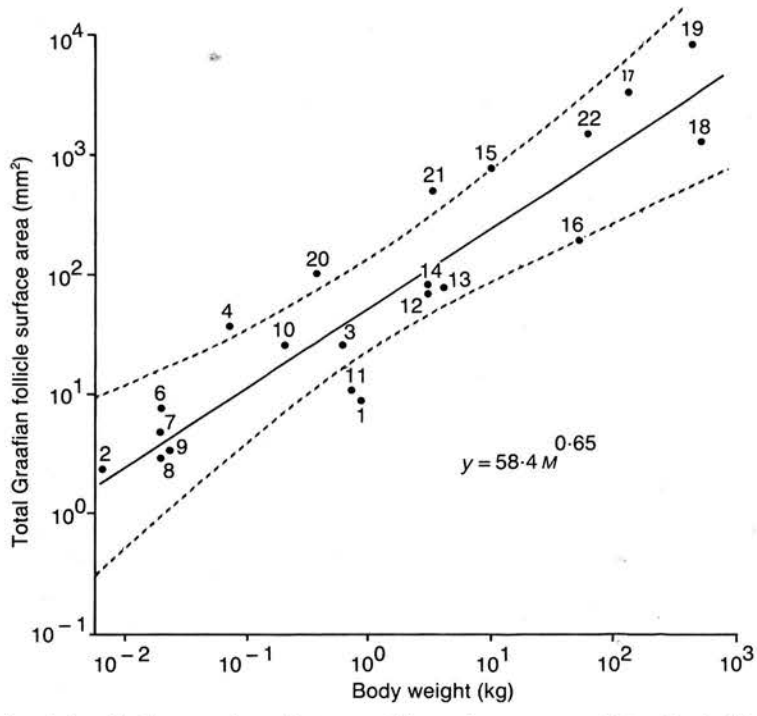

FIG. 5. Allometric relationship between the surface area of the ovulatory quota of Graafian follicles and body weight (see Table I for species code list for Figs 1-6).

weight could account for most of the variation in Graafian sizes and was fitted by the allometric formula ($r^2 > 0{\cdot}75$) (Table II).

A greater proportion of the variation in follicular sizes was encompassed by the allometric relationship when the dimensions of a set of preovulatory follicles were combined rather than considered singly, this being indicated by a rise in r^2 from 0·75 to 0·85 (Table II). The impact of this manoeuvre was attributed to the exceptional data from the Plains viscacha more than any other single species, since this rodent, which weighs 2–4 kg, combines a high ovulation rate with diminutive follicular size. Apart from this interactive effect and the negative relationship to Graafian size, the ovulation rate was not correlated significantly with the other variables being studied. The allometric exponents for total Graafian surface areas and volumes were $0{\cdot}651 \pm 0{\cdot}063$ and $1{\cdot}056 \pm 0{\cdot}103$, respectively; the second of these being not significantly different from unity. Graafian dimensions in the three species of primates were, in most of the comparisons made, significantly greater than would be expected on the basis of body weight (Figs 5, 6).

Discussion

The results demonstrate, *inter alia*, that most variation between species in the sizes of Graafian follicles and, to a lesser extent, of primordial follicles can be accounted for by differences in adult body weight. The scaling relationships are consistent with the general allometric formula, $y = aM^b$. In contrast to the follicle and to the spermatozoan (Cummins & Woodall, 1985),

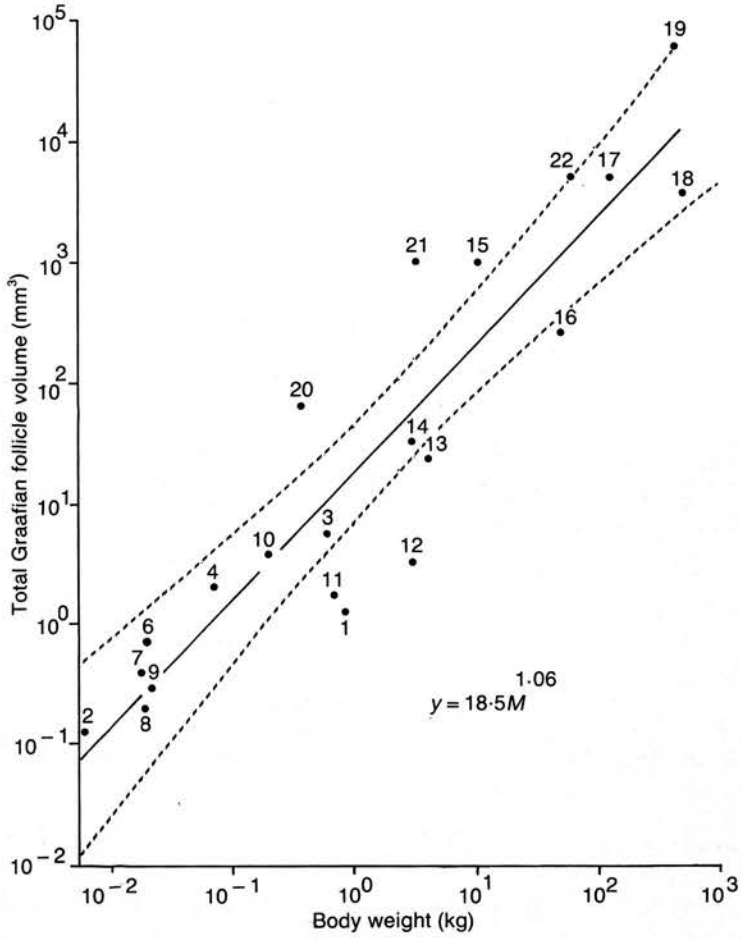

FIG. 6. Allometric relationship between the volume of the ovulatory quota of Graafian follicles and body weight.

which is scaled negatively with body weight, oocytes of eutherian mammals were relatively similar in size, thus confirming the results of earlier studies (Austin, 1976). Attempts have been made to find physiological explanations for the allometric expressions obtained, but, first, the principal assumptions underlying the study will be discussed.

The value of allometric formulae as devices for describing general relationships between two variables and for predicting one variable from the other rests on the assumptions that the data are taken from a representative sample of species and of individuals and that measurements are reliable. The present sample was restricted by the availability of specimens of sufficient quality for providing most, if not all, of the seven measurements sought; consequently, it includes a number of domesticated species and laboratory stocks. There were no indications, however, of systematic differences in the raw data obtained from these animals compared with other species. The list of species represents nine taxonomic orders and the body weights range from 0·005–500 kg with order of magnitude differences in Graafian follicle sizes. Such large differences within

correlated variables, in combination with logarithmic transformation of data, reduces the impact of individual variation on the fitting of a straight line by regression analysis. Compared with that arising from measurement errors, a greater proportion of the total variation is probably due to that existing naturally within species and even within ovaries in the size and numbers of preovulatory follicles.

Allometric variation of Graafian follicle sizes is perhaps the most notable finding in this study. It would appear to be due to variation in the volume of follicular fluid, since the thickness of the *membrana granulosa* was relatively constant. Allometric scaling has presumably evolved to adapt follicular function to the changing physiological conditions of increased body size. Ovarian follicles have two major functions: they support and control the maturation of oocytes, and secrete hormones which integrate sexual physiology and behaviour. Since the range of oocyte size was small, Graafian dimensions might be expected to subserve the second function.

On *a priori* grounds, a more significant correlation would be anticipated between body weight and Graafian dimensions when considered collectively rather than individually, since follicles are integrated in development for ovulation and for secretion of hormones into the body pool. This expectation was confirmed, but additional data are required to determine whether an interactive effect of numbers and sizes of preovulatory follicles exists continuously across a wide range of species, or is confined to species with very high ovulation rates where the importance of scaling is obvious. In the Plains viscacha (*Lagostomus maximus*) and the Elephant shrew (*Elephantulus myurus*), ovulation rates vary from 200–800 and from 50–180, respectively (Weir, 1971; Tripp, 1971), which appears to have affected Graafian sizes in accordance with theory. The norms of body mass based on a regression line for the other species would predict that their Graafian volumes would be 22·0 and 0·23 mm^3 instead of the observed values of 0·01 and 0·02 mm^3. The ovarian mass and surface area required for accommodating and ovulating the predicted follicle sizes in such large numbers may not be physiologically realistic.

The hypothesis that the total surface area presented for secretion by a set of preovulatory follicles matches the distribution volume for oestrogen and other hormones is not supported by the present results, since a hypoallometric exponent of 0·65 was obtained. Plasma oestrogen levels are independent of body weight. The exponent suggests a closer relationship to the body surface area ($M^{0·67}$) than to body volume ($M^{1·0}$). The isometric variation between the volumes of Graafian follicles and body weight, of which the hormone distribution volume is a fixed proportion (Stahl, 1967), would be expected if follicles consisted entirely of a homogeneous population of secretory cells. The majority of space in most species is, however, extracellular, and there is only a shell of theca and granulosa cells, with most metabolic activity confined to the periphery (Gosden & Byatt-Smith, 1986). Isometric variation is therefore unexpected, unless the follicular fluid, which contains high concentrations of steroid hormones (Edwards, 1974), acts as a hormone store and is dynamically involved in the maintenance of circulating hormone levels. Variation in Graafian size might also be explained, in principle, on the basis that the maximum dimensions determine the size of the corpora lutea. Being vascular and relatively more solid than the follicles, corpora lutea might be expected to vary isometrically with the distribution volume. This hypothesis may, however, not fully account for their dimensions because of the existence of accessory corpora lutea and of heterogeneous luteal cell populations in some species (Schwall & Niswender, 1985). Isometric relationships have not been found among other endocrine organs (Brody, 1945; Harcourt *et al.*, 1981), and there is therefore no general rule of fixed apportionment of organ space within the abdomen. On the basis of the present information,

there would appear to be little support for the view that simple geometrical scaling factors dictate follicular sizes, possibly because of the complexity of physiological factors.

Besides providing a general description of scaling relationships, allometric expressions can also draw attention to exceptional data and, hence, indicate peculiar physiological or anatomical features. In primates, the disproportionately large size of Graafian follicles is of particular interest when considered in relation to the remarkably high levels of oestradiol-17β during the preovulatory phase. The levels are about 3–10 fold greater than those in non-primate species which are invariably $< 0{\cdot}5$ pmol ml^{-1} (e.g. for non-primates: Scaramuzzi, Caldwell & Moor, 1970; Noden, Oxender & Hafs, 1975; Smith, Freeman & Neill, 1975; Austad, Lunde & Sjaastad, 1976; Hodges, Henderson & McNeilly 1983; and for primates: Weick *et al.*, 1973; Korenman *et al.*, 1974; Reyes, Winter, Faiman & Hobson, 1975; Nadler, Graham, Collins & Gould, 1979). Such notable differences are not conspicuous among the other gonadal steroids in either females or males. They cannot reflect simply a distinction between those species having menstrual cycles and those which do not, since especially high levels of oestradiol are present among the New World monkeys, which do not menstruate (Wolf, O'Connor & Robinson, 1977; Harlow, Hearn & Hodges, 1984). The elevated oestrogen levels in primates could be due to larger follicles. It is equally possible, however that they reflect a slower rate of clearance from the circulation, resulting from greater plasma sex steroid binding activity and consequent protection from metabolism (Corvol & Bardin, 1973; Siiteri *et al.*, 1982).

The norms of Graafian follicle scaling may not be obeyed by some bats. This tentative conclusion is based on limited information gained from another species because the ovaries of pipistrelle bats were anoestrus. The ovaries of the Lesser horseshoe bat (*Rhinolophus hipposideros*) contains one or two Graafian follicles which measure 0·3 mm in diameter (Harrison Matthews, 1937). The follicular volume in this bat, although similar to that of terrestrial mammals of similar size (e.g. common shrew), is considerably less than that predicted by allometry for the known ovulatory quota.

The present study has been mainly concerned with the effects of interspecific rather than intraspecific differences in body weight, because the latter were comparatively small in the samples obtained. It is known from studies of other variables that allometric relationships across a range of species may not apply within any given species (Calder, 1984), and there is tentative evidence of this qualification with respect to follicles. The Merino sheep has a mean ovulation rate of 1·2, with Graafian follicles measuring 8 mm, whereas in the highly fecund Booroola breed the values are 5·2 and 4 mm (Driancourt, Cahill & Bindon, 1985). The total Graafian surface areas and volumes are therefore 241 and 261 mm^2 and 321 and 174 mm^3, respectively, in the two breeds. These findings contradict the expectation that the total volumes should be the same.

The allometric relationship observed for Graafian follicles cannot be simply accounted for by scaling up from that observed among primordial follicles, since different compartments are involved in the two types, namely the ooplasm and the antrum. A physiological basis for scaling of primordial follicle sizes and for the exceptional cases, rabbits and cats, is even more obscure than that for Graafian follicles. Primordial follicle size was significantly correlated with several of the other variables, but these relationships are unlikely to be causal.

In contrast to the scaling of follicle sizes at either end of the growing phase, the morphogenetic transition from the preantral to the antral stage occurs at a similar size in all species. In mice, it is initiated when 2000–3000 granulosa cells have accumulated and, as cell volume is approximately the same, it is probably similar in other animals (unpubl. obs.). In a few species, follicles mature without forming an antrum (Nicholl & Racey, 1985), but the dimensions hardly exceed

the upper size limit for solid follicles in others. The striking uniformity of size at this stage is yet another puzzling observation without a clear explanation at present. Experimental studies are needed to test whether it reflects the physical limitations of diffusion of respiratory gases or metabolites, or represents a developmental requirement for accumulating a critical number of granulosa cells for commitment to separate pathways in the Graafian follicle.

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REFERENCES

Altman, P. L. & Dittmer, D. S. (Ed.) (1972). *Biology data book* **1.** 2nd edn. Bethesda, MD: Fed. Am. Socs Exp. Biol.

Austad, R., Lunde, A. & Sjaastad, Ø. V. (1976). Peripheral plasma levels of oestradiol-17β and progesterone in the bitch during the oestrous cycle, in normal pregnancy and after dexamethasone treatment. *J. Reprod. Fert.* **46:** 129–136.

Austin, C. R. (1976). Specialization of gametes. In *Reproduction in mammals. Book 6. The evolution of reproduction*: 149–182. Austin, C. R. & Short, R. V. (Eds). Cambridge: Cambridge University Press.

Brody, S. (1945). *Bioenergetics and growth.* New York: Reinhold.

Calder III, W. A. (1984). *Size, function and life history.* Cambridge, MA: Harvard University Press.

Corvol, P. & Bardin, C. W. (1973). Species distribution of testosterone-binding globulin. *Biol. Reprod.* **8:** 277–282.

Cummins, J. M. & Woodall, P. F. (1985). On mammalian sperm dimensions. *J. Reprod. Fert.* **75:** 153–175.

Driancourt, M. A., Cahill, L. P. & Bindon, B. M. (1985). Ovarian follicular populations and preovulatory enlargement in Booroola and control Merino ewes. *J. Reprod. Fert.* **73:** 93–107.

Edwards, R. G. (1974). Follicular fluid. *J. Reprod. Fert.* **37:** 189–219.

Gosden, R. G. & Byatt-Smith, J. G. (1986). Oxygen concentration gradient across the ovarian follicular epithelium: model, predictions and implications. *Human Reprod.* **1:** 65–68.

Gosden, R. G. & Telfer, E. (1987). Numbers of follicles and oocytes in mammalian ovaries and their allometric relationships. *J. Zool., Lond.* **211.**

Harcourt, A. H., Harvey, P. H., Larson, S. G. & Short, R. V. (1981). Testis weight, body weight and breeding system in primates. *Nature, Lond.* **293:** 55–57.

Harlow, C. R., Hearn, J. P. & Hodges, J. K. (1984). Ovulation in the marmoset monkey: endocrinology, prediction and detection. *J. Endocr.* **103:** 17–24.

Harrison Matthews, L. (1937). The female sexual cycle in the British horseshoe bats, *Rhinolophus ferrum-equinum insulanus* Barrett-Hamilton and *R. hipposideros minutus* Montagu. *Trans. zool. Soc. Lond.* **23:** 224–255.

Hodges, J. K., Henderson, C. & McNeilly, A. S. (1983). Circulating oestrogen concentrations during pregnancy in the African elephant (*Loxodonta africana*). *J. Reprod. Fert.* **67:** 121–127.

Korenman, S. G., Stevens, R. H., Carpenter, L. A., Robb, M., Niswender, G. D. & Sherman, B. M. (1974). Estradiol radioimmunoassay without chromatography: procedure, validation and normal values. *J. Clin. Endocr. Metab.* **38:** 718–720.

May, R. M. & Rubenstein, D. I. (1984). Reproductive strategies. In *Reproduction in mammals. Book 4. Reproductive fitness*: 1–23. 2nd edn. Austin, C. R. & Short, R. V. (Eds). Cambridge: Cambridge University Press.

Nadler, R. D., Graham, C. E., Collins, D. C. & Gould, K. G. (1979). Plasma gonadotropins, prolactin, gonadal steroids, and genital swelling during the menstrual cycle of lowland gorillas. *Endocrinology* **105:** 290–296.

Nicholl, M. E. & Racey, P. A (1985). Follicular development, ovulation, fertilization and fetal development in tenrecs (*Tenrec ecaudatus*). *J. Reprod. Fert.* **74:** 47–55.

Noden, P. A., Oxender, W. D. & Hafs, H. D. (1975). The cycle of oestrus, ovulation and plasma levels of hormones in the mare. *J. Reprod. Fert. (Suppl.)* **23:** 189–192.

Parkes, A. S. (1932). Size of Graafian follicle at ovulation. *Proc. R. Soc. (B)* **109:** 185–213.

Reyes, F. I., Winter, J. S. D., Faiman, C. & Hobson, W. C. (1975). Serial serum levels of gonadotropins, prolactin and sex steroids in the nonpregnant and pregnant chimpanzee. *Endocrinology* **96:** 1447–1455.

Scaramuzzi, R. J., Caldwell, B. V. & Moor, R. M. (1970). Radioimmunoassay of LH and oestrogen during the oestrous cycle of the ewe. *Biol. Reprod.* **3:** 110–119.

Schwall, R. H. & Niswender, G. D. (1985). Two types of steroidogenic luteal cells in the ewe: morphological and biochemical characteristics. In *Implantation of the human embryo*: 31–38. Edwards, R. G., Purdy, J. M. & Steptoe, P. C. (Ed.). London & Orlando: Academic Press.

Siiteri, P. K., Murai, J. T., Hammond, G. L., Nisker, J. A., Raymoure, W. J. & Kuhn, R. W. (1982). The serum transport of steroid hormones. *Rec. Prog. Horm. Res.* **38:** 457–503.

Smith, M. S., Freeman, M. E. & Neill, J. D. (1975). The control of progesterone secretion during the estrous cycle and early pseudopregnancy in the rat: prolactin, gonadotropin and steroid levels associated with rescue of the corpus luteum of pseudopregnancy. *Endocrinology* **96:** 219–226.

Stahl, W. R. (1967). Scaling of respiratory variables in mammals. *J. appl. Physiol.* **22:** 453–460.

Tripp, H. R. H. (1971). Reproduction in elephant-shrews (Macroscelididae) with special reference to ovulation and implantation. *J. Reprod. Fert.* **26:** 149–159.

Weick, R. F., Dierschke, D. J., Karsch, F. J., Butler, W. R., Hotchkiss, J. & Knobil, E. (1973). Periovulatory time courses of circulating gonadotropic and ovarian hormones in the rhesus monkey. *Endocrinology* **93:** 1140–1147.

Weir, B. J. (1971). The reproductive organs of the female plains viscacha, *Lagostomus maximus*. *J. Reprod. Fert.* **25:** 365–373.

Wolf, R. C., O'Connor, R. F. & Robinson, J. A. (1977). Cyclic changes in plasma progestins and estrogens in squirrel monkeys. *Biol. Reprod.* **17:** 228–231.